

On the Mechanism of Action of Aspartate Aminotransferase

Inaugural-Dissertation

zur

Erlangung der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von
HANS PETER TOBLER
aus Zollikon / ZH

Begutachtet von Herrn Prof. Dr. Ph. Christen

Zürich 1986
Zentralstelle der Studentenschaft

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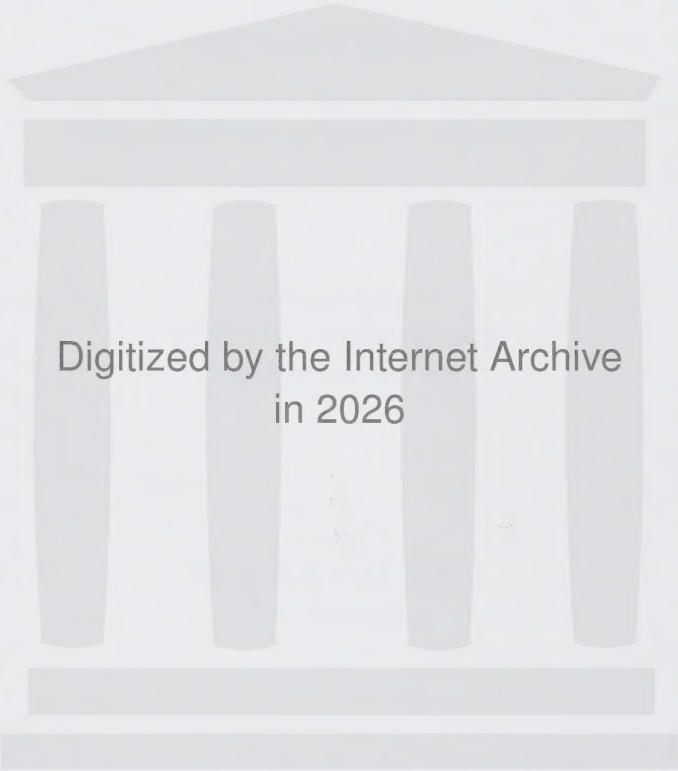
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Meiner Frau Susi sowie
meinen Kindern Ursina
und Seraina gewidmet

DANK

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Parts of this work have already been presented in preliminary form:

- Apoenzyme-catalyzed model reactions for enzymic transamination. Tobler, H.P., Gehring, H., and Christen, P. (1984) Abstracts of the 16th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Zurich, *Experientia* 40, 614.
- Stereospecific Labilization of the C4'-pro-S Hydrogen of Pyridoxamine 5'-Phosphate in Aspartate Aminotransferase. Tobler, H.P., Gehring, H., and Christen, P. (1986) Abstracts of the 18th Annual Meeting of the Swiss Societies for Experimental Biology (USGEB), Basle, in press.

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The following publications are in preparation:

- Apoenzyme-catalyzed Model Reaction for Aspartate Aminotransferase. Tobler, H.P., Gehring, H., and Christen, P.
- Stereospecific Labilization of the C4'-pro-S Hydrogen of Pyridoxamine 5'-Phosphate in Aspartate Aminotransferase. Activators and Inhibitors. Tobler, H.P., Gehring, H., and Christen, P.

General footnotes

The abbreviations used are: PL, pyridoxal; PLP, pyridoxal 5'-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5'-phosphate; PNP, pyridoxine 5'-phosphate; P_i , inorganic phosphate; HEPES, N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC, high performance anion exchange chromatography.

Aspartate aminotransferase is an α_2 dimer with functionally identical subunits (Schlegel et al., 1977). In this study, the indicated concentrations of enzyme and all values normalized with respect to enzyme concentration refer to the concentration of subunits rather than enzyme dimer.

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SUMMARY

The first part of this thesis establishes an apoenzyme-catalyzed model reaction for aspartate aminotransferase by means of which the *de novo* formation of the external aldimine from pyridoxal and amino acid substrate was compared kinetically with its generation by transamination from a pre-existing internal aldimine between pyridoxal and Lys 258. The second part reports that aspartate aminotransferase after reconstitution with [4'-³H]pyridoxamine 5'-phosphate catalyzes the stereospecific release of its pro-S ³H into the solvent. The proton which is thus exchanged in the absence of substrate is the same that is involved in the tautomerization of the ketimine to the aldimine intermediate during the reaction of the pyridoxamine 5'-phosphate form of the enzyme with an oxo acid substrate.

Apoenzyme-catalyzed Model Reaction for Aspartate Aminotransferase

The apoenzyme of aspartate aminotransferase catalyzes the reaction $\text{pyridoxal} + \text{aspartate} \rightleftharpoons \text{pyridoxamine} + \text{oxalacetate}$ with the nonphosphorylated cofactor acting as a dissociable substrate rather than as a prosthetic group (Wada, H., and Snell, E.E. (1962) J. Biol. Chem. 237, 127-132). In this model system the two half-reactions of transamination can be monitored separately. In the case of mitochondrial aspartate aminotransferase from chicken the rates of the forward and the back reaction are 0.13% and 0.005%, respectively, of that of the rate of the holoenzyme-catalyzed transamination with aspartate and 2-oxoglutarate as substrates. The forward reaction was used in an attempt to reassess a long-standing question in the study of enzymic pyridoxal 5'-phosphate-dependent reactions, i.e., to

assess the catalytic advantage of replacing the *de novo* formation of the coenzyme substrate aldimine intermediate ("external" aldimine) by the transformation of the pre-existing "internal" aldimine between the coenzyme and the ϵ -amino group of Lys 258 into the external aldimine *via* a geminal diamine.

Apoaspartate aminotransferase that has been freed from the tightly bound inorganic phosphate ion (Iriarte, A., Kraft, K., and Martinez-Carrion, M. (1985) J. Biol. Chem. 260, 7457-7463) readily forms an internal aldimine on addition of pyridoxal. Phosphate-free apoenzyme was prepared by displacing the phosphate ion with hydrogen carbonate or phosphite, or by high performance anion exchange chromatography. The phosphate-containing apoenzyme cannot accommodate pyridoxal bound as internal aldimine. In the case of the phosphate-free apoenzyme, the transamination reaction initiated by the addition of aspartate starts with a burst of pyridoxamine formation. This burst follows first-order kinetics and represents the rapid decay of the preformed internal aldimine. In the case of the apoenzyme containing tightly bound phosphate, transamination proceeds from the start *via de novo* formation of the external aldimine. In this reaction, the apparent K_m value for pyridoxal (2.1 mM) is considerably higher than in the case of the phosphate-free apoenzyme (80 μ M). However, the maximum rate of pyridoxamine formation at saturating concentrations is the same for both phosphate-containing and phosphate-free apoenzyme ($k_{cat} = 12 \text{ min}^{-1}$). Apparently, neither the transaldimination step in the case of the phosphate-free apoenzyme nor the *de novo* formation of the external aldimine in the case of the phosphate-liganded apoenzyme under saturating concentrations of pyridoxal are rate-limiting in the apoenzyme-catalyzed transamination of pyridoxal and aspartate. The data obtained with the present model system thus do not support but also not exclude the notion of

a kinetic advantage brought about by the existence of the internal aldimine bond between pyridoxal and the enzyme protein.

Stereospecific Labilization of the C4'-pro S Hydrogen of Pyridoxamine 5'-Phosphate in Aspartate Aminotransferase

In the course of a half reaction of enzymic transamination the aldimine adduct formed between the coenzyme pyridoxal 5'-phosphate and the amino acid substrate tautomerizes to the ketimine intermediate which is then hydrolyzed to the oxo acid product and the pyridoxamine 5'-phosphate form of the enzyme. In the reverse half-reaction the tautomerization is initiated by the removal of a proton from the pro-S position at C4' of the pyridoxamine 5'-phosphate moiety of the ketimine intermediate. The second part of this thesis investigates the question whether the pro-S hydrogen at C4' of pyridoxamine 5'-phosphate is labilized by its active site environment independently of the formation of the ketimine intermediate, i.e. in the absence of substrate.

Reconstitution of apoaspartate aminotransferase (mitochondrial isoenzyme from chicken) with [4'-³H]pyridoxamine 5'-phosphate results indeed in a stereospecific exchange of pro-S ³H with solvent water. The exchange follows first order kinetics ($t_{1/2} = 23$ min at pH 7.5 and 25°C) and is 32,000 times faster than that from free coenzyme. Rigorous control experiments excluded the possibility that the observed exchange was due to a transamination reaction of the enzyme with contaminating oxo acid substrates. Enzyme-bound [³H]-pyridoxine 5'-phosphate is not detritiated suggesting an essential role of the 4'-amino group and its hydrogen bond to the ε-amino group of Lys 258.

Formation of the unproductive complex of the [^3H]pyridoxamine 5'-phosphate-enzyme with aspartate and glutamate results in a 430fold acceleration of ^3H release. The rate of deprotonation as estimated by correcting for the isotope effect thus becomes only 2 orders of magnitude slower than the reaction rate becoming only 2 to 3 orders of magnitude slower than the rate of the overall transamination reaction. In contrast, addition of borohydride or cyanoborohydride immediately stops ^3H release. Apparently, these compounds or derivatives thereof form relatively stable complexes probably with amino or hydroxyl groups of the protein and the cofactor, thus preventing ^3H release either directly or by interfering with conformational features of the enzyme. Experiments with differential chemical modifications and with a fluorescent reporter group indicate that the activating effect of aspartate or glutamate on the release of ^3H is accompanied by a shift of the so-called open/closed conformational equilibrium of the enzyme (Kirsch, J.F., Eichele, G., Ford, G.C., Vincent, M.G., Jansonius, J.N., Gehring, H., and Christen, P. (1984) *J. Mol. Biol.* 174, 497-525) toward the closed conformation; the inhibiting effect of borohydride and cyanoborohydride appears to be accompanied by a shift toward the open conformation. The data thus suggest that the efficiency of at least part of the catalytic apparatus of aspartate aminotransferase becomes fully operative only in the closed conformation of the enzyme.

Measurement of ^3H release from enzyme-bound [^3H]pyridoxamine 5'-phosphate provides an experimental means to probe particular aspects of the catalytic competence of aspartate aminotransferase, i.e. the acid/base properties of C4' and the modulating effects of its active site environment, independently of substrate turnover.

ZUSAMMENFASSUNG

Im ersten Teil der vorliegenden Dissertation wird eine Apoenzym-katalysierte Modellreaktion der Aspartat-Aminotransferase vorgestellt, bei welcher die *de novo* Bildung des externen Aldimins aus Pyridoxal und dem Aminosäuresubstrat kinetisch mit dessen Entstehung durch Transiminierung aus dem vorgebildeten internen Aldimin zwischen Pyridoxal und Lys 258 verglichen wird. Der zweite Teil berichtet über die nach Rekonstitution der Aspartat-Aminotransferase mit [4'-³H]Pyridoxamine-5'-phosphat einsetzende stereospezifische Freisetzung seines pro-S ³H ins Lösungsmittel. Das in Abwesenheit von Substrat ausgetauschte Proton ist dasselbe, welches in die Tautomerisierung der Ketimin- zur Aldiminzwischenverbindung während der Reaktion der Pyridoxamin-5'-phosphat-Form des Enzyms mit dem Ketosäuresubstrat einbezogen ist.

Apoenzym-katalysierte Modell-Reaktion für die Aspartat-Aminotransferase

Das Apoenzym der Aspartat-Aminotransferase katalysiert die Reaktion $\text{Pyridoxal} + \text{Aspartat} \rightleftharpoons \text{Pyridoxamin} + \text{Oxalacetat}$. Dabei agiert der nichtphosphorylierte Cofaktor als dissoziierbares Substrat und nicht als prosthetische Gruppe (Wada, H., and Snell, E.E. (1962) J. Biol. Chem. 237, 127-132). Dieses Modellsystem schafft die Möglichkeit, beide Halbreaktionen getrennt voneinander verfolgen zu können. Im Falle des mitochondrialen Isoenzym der Aspartat-Aminotransferase des Huhns beträgt die Geschwindigkeit der Hin- und Rückreaktion 0.13% bzw. 0.005% der Geschwindigkeit der vom Holoenzym katalysierten Transaminierung mit Aspartat und α -Ketoglutarat. Die Hinreaktion wurde näher untersucht, um eine langanstehende

Frage in der Erforschung der enzymatischen Pyridoxal-5'-phosphat-abhängigen Reaktionen anzugehen, nämlich den katalytischen Vorteil der Transformierung des "internen" Aldimins zwischen dem Coenzym und der ϵ -Aminogruppe von Lys 258 *via* geminales Diamin gegen die *de novo* Bildung der Coenzym-Substrat-Aldimin-Zwischenverbindung ("externes" Aldimin) abzuschätzen.

Die Apoaspartat-Aminotransferase enthält an der aktiven Stelle ein fest gebundenes Phosphation (Iriarte, A., Kraft, K., and Martinez-Carrion, M. (1985) J. Biol. Chem. 260, 7457-7463). Von Phosphat befreites Apoenzym ist fähig, mit zugegebenem Pyridoxal das interne Aldimin zu bilden; im Gegensatz dazu ist das phosphathaltige Apoenzym hierzu unfähig. Im Falle des phosphatfreien Enzyms mit vorgebildeter interner Schiffscher Base beginnt die Transaminierungsreaktion nach Zugabe von Aspartat mit einem "burst" der Pyridoxamin-Bildung. Dieser "burst" verläuft nach einer Kinetik erster Ordnung und entspricht dem schnellen Zerfall des internen Aldimins. Andererseits verläuft die Transaminierung mit phosphathaltigem Enzym *via de novo* Bildung zum externen Aldimin. In dieser Reaktion ist der apparente K_m -Wert für Pyridoxal mit 2.1 mM beträchtlich höher als im phosphatfreien Apoenzym (K_m 80 μ M). Dagegen ist die maximale Geschwindigkeit der Bildung von Pyridoxamin unter Sättigungsbedingungen für beide Enzyme gleich gross ($k_{cat} = 12 \text{ min}^{-1}$). Offenbar sind weder Transaldiminierung im Falle des phosphatfreien Enzyms noch die *de novo* Bildung beim phosphathaltigen Enzym unter Sättigungskonzentrationen von Pyridoxal geschwindigkeitsbestimmend in der Apoenzym-katalysierten Transaminierung mit Pyridoxal und Aspartat. Die mit dem vorgestellten Modellsystem erhaltenen Ergebnisse stützen zwar nicht die Hypothese eines kinetischen Vorteils des Vorkommens des internen Aldimins, sie schliessen diese Möglichkeit aber auch nicht aus.

Stereospezifische Labilisierung des C4'-pro-S Wasserstoffs des Pyridoxamin 5'-Phosphats durch die Aspartat-Aminotransferase

Im Verlauf einer Halbreaktion der enzymatischen Transaminierung tautomerisiert das externe Aldimin zur Ketimin-Zwischenverbindung, welche darauf zum Ketosäureprodukt und der Pyridoxamin-5'-phosphat-Form des Enzyms hydrolysiert wird. In der Rückreaktion wird die Tautomerisierung durch das Entfernen des pro-S Protons in der C4'-Position des Pyridoxamin-5'-phosphat-Teils der Ketimin-Zwischenverbindung eingeleitet. Die vorliegende Studie hat die Frage aufgegriffen, ob der pro-S Wasserstoff am C4' von Pyridoxamin-5'-phosphat an der aktiven Stelle des Enzyms unabhängig von der Ketimin-Bildung, d.h. bereits in der Abwesenheit von Substrat labilisiert wird.

Die Rekonstitution der Aspartat-Aminotransferase (mitochondriales Isoenzym) mit [4'-³H]Pyridoxamin-5'-phosphat hat eine stereospezifische Freisetzung des pro-S ³H ins Lösungsmittel zur Folge. Dieser Austausch mit Wasserstoff gehorcht einer Kinetik erster Ordnung ($t_{1/2} = 23$ min bei pH 7.5 und 25°C) und ist 32'000mal schneller als jener von freiem Coenzym. Eingehende Kontrollexperimente schlossen die Möglichkeit der beobachteten ³H-Freisetzung durch Transaminierung des Enzyms mit eventuell kontaminierenden Ketosäuren aus. Enzymgebundenes [³H]Pyridoxin-5'-phosphat wird nicht detritiert; offenbar ist die 4'-Aminogruppe und ihre Wasserstoffbindung zur ϵ -Aminogruppe von Lys 258 wichtig für den Austausch.

Die Bildung des unproduktiven Adsorptionskomplexes der Pyridoxamin-5'-phosphat-Form des Enzyms mit Aspartat oder Glutamat führt zu einer markanten Beschleunigung (maximal 430fach) der ³H-Freisetzung. Damit wird die Geschwindigkeit der Deprotonierung, soweit man sie durch Korrektur für den Isotopen - Effekt abschätzen kann, nur 2 Zehnerpotenzen kleiner als diejenige der normalen Transaminierungs-

gesamtreaktion. Im Gegensatz dazu sistiert die Zugabe von Borhydrid oder Cyanborhydrid sofort die Freisetzung von ^3H . Diese Inhibitoren bilden offenbar relativ stabile Komplexe, vermutlich mit Amino- oder Hydroxylgruppen des Proteins und des Cofaktors und verhindern so entweder direkt oder über eine Veränderung der konformationellen Eigenschaften des Enzyms die ^3H -Freisetzung. Experimente mit differentiellen chemischen Modifikationen und mit einer fluoreszierenden Reportergruppe zeigen, dass der aktivierende Effekt von Aspartat oder Glutamat auf die ^3H -Freisetzung von einer Verschiebung des sogenannten offen/geschlossen Konformationsgleichgewichts des Enzyms (Kirsch, J.F., Eichele, G., Ford, G.C., Vincent, M.G., Jansonius, J.N., Gehring, H., and Christen, P. (1984) J. Mol. Biol. 174, 497-525) nach der geschlossenen Konformation begleitet ist; die hemmende Wirkung von Borhydrid und Cyanborhydrid scheint dagegen von einer Verschiebung gegen die offene Konformation begleitet zu sein. Die Beobachtung lässt die Vermutung zu, dass die Effizienz des katalytischen Apparats der Aspartat-Aminotransferase mit der geschlossenen Konformation gekoppelt ist.

Messung der ^3H -Freisetzung von enzymgebundenem [^3H]Pyridoxamin-5'-phosphat stellt eine neue experimentelle Möglichkeit dar, bestimmte Aspekte der katalytischen Wirkung der Aspartat-Aminotransferase, wie die Säure/Base Eigenschaften von C4' als auch modulierende Effekte von dessen Umgebung an der aktiven Stelle, unabhängig von Substratumsatz zu untersuchen.

I. GENERAL INTRODUCTION

1. Molecular Properties of Aspartate Aminotransferase

Aspartate aminotransferase is the most extensively investigated representative of the large group of PLP (vitamin B₆)-dependent enzymes which catalyze manyfold reactions in the metabolism of amino acids. In all these enzymes, PLP is not present as free aldehyde but is bound covalently *via* a Schiff base linkage to an active site lysine residue (Hughes et al., 1962). Aspartate aminotransferase catalyzes the transfer of the α -amino group of aspartate or glutamate to the oxo acids 2-oxoglutarate or oxalacetate, respectively. The reaction follows a double displacement mechanism during which the coenzyme shuttles between the PLP form and the PMP form (see Fig. 1, A and F). In the forward half-reaction of transamination, PLP reacts with the amino acid substrate forming PMP and the corresponding oxo acid product. In the reverse half-reaction, PMP reacts with the 2-oxo acid substrate regenerating PLP and forming the amino acid product. The general mechanism of vitamin B₆-catalysis in transamination was first proposed by Braunstein and Shemyakin (1953) and by Metzler, Ikawa and Snell (1954).

In animal cells, two homologous, heterotopic aspartate aminotransferases are found, i.e. a cytosolic and a mitochondrial isoenzyme. Both aspartate aminotransferases are coded for by nuclear DNA, and are synthesized in the cytosol, the mitochondrial isoenzyme as a precursor with higher molecular weight (Sonderegger et al., 1980) on free polysomes (Sonderegger et al., 1982). The translocation of the precursor from the cytosol into the mitochondria depends on an energized inner mitochondrial membrane and a metal-dependent protease in the mitochondrial matrix for processing (Behra and Christen, 1986). In

two species, chicken and pig, the amino acid sequences of both the mitochondrial and cytosolic isoenzyme have been determined (Ovchinnikov et al., 1973; Doonan et al., 1980; Shlyapnikov et al., 1979; Barra et al., 1980; Kagamiyama et al., 1980; Graf-Hausner et al., 1983). The degree of inter-species similarity of the mitochondrial aspartate aminotransferases (86%, chicken - pig) and of the cytosolic aspartate aminotransferases (83%, chicken - pig) by far exceeds the intra-species similarity of the mitochondrial and cytosolic isoenzyme (46% chicken; 48% pig). In accordance with the degree of amino acid sequence identity between cytosolic and mitochondrial aspartate aminotransferase, the chain folding patterns of the two isoenzymes proved virtually superimposable, with the exception of a few deletions in the mitochondrial isoenzyme. Recently, the cDNAs of the precursors of mitochondrial aspartate aminotransferase from pig and chicken have been molecularly cloned (Joh et al., 1985; Jaussi et al., 1985).

Aspartate aminotransferase is composed of two identical subunits. The two active sites are situated on opposite sides of the enzyme dimer in clefts near the subunit interface. Each subunit is composed of a large coenzyme binding domain and a small domain. X-Ray crystallographic analysis of both the mitochondrial (Ford et al., 1980; Kirsch et al., 1984) and the cytosolic isoenzyme (Borisov et al., 1980; Arnone et al., 1982; Harutyunyan et al., 1982) has shown that the crystalline enzyme exists in two different conformational states, designated the "open" and the "closed" form. The two forms differ in the relative position of the small and the large domain. The open form is characterized by a wide active site crevice. In the closed conformation the small domain has moved toward the active site and has narrowed the crevice.

Extensive studies with aspartate aminotransferase in solution, i.e. differential chemical modification of sulfhydryl groups (Birchmeier et al., 1973; Gehring and Christen, 1978), differential proteolytic modification (Sandmeier and Christen, 1980; Meer and Gehring, 1984), measurement of peptide hydrogen-deuterium exchange (Pfister et al., 1978, 1985) have shown that the enzyme undergoes substantial changes in conformation during the transamination reaction. In the case of the mitochondrial isoenzyme, the reactivity of Cys 166 has proven a valuable indicator of conformational changes of the protein matrix (Gehring and Christen, 1978). Cys 166 is situated on the tip of the PLP-binding domain, 25 Å away from the coenzyme at the interface with the small domain (Kirsch et al., 1980). Its reactivity toward modifying reagents is increased when enzyme substrate intermediates are formed, signaling a syncatalytic shift of the open/closed equilibrium toward the closed conformation.

The relative values of the conformational parameters that characterize a given functional state of the enzyme agree with each other to a notable extent despite important differences among the various methods used for monitoring the conformation of the enzyme. The parametric increments signaling a conformational change correspond in two cases, i.e. in peptide hydrogen exchange and proteolytic cleavage, to a decrease in reactivity, and in the other case, i.e. chemical modification of Cys 166, to an increase in reactivity. The correlation among the various conformational parameters characterizing a given functional state indicates that all methods monitor the same process, i.e. shifts in the same conformational equilibrium.

The X-ray crystallographic studies of mitochondrial aspartate aminotransferase have detected also only one conformational change, i.e. the bulk movement of the small domain in the open/closed equilibrium. Apparently, shifts in one and the same

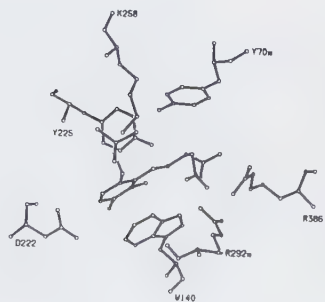
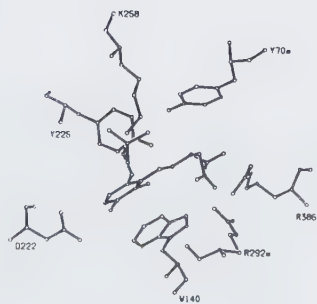
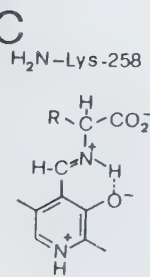
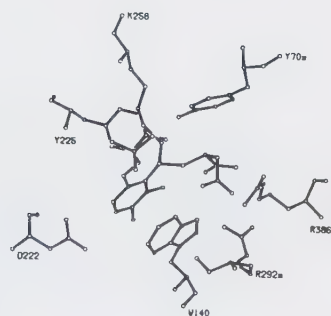
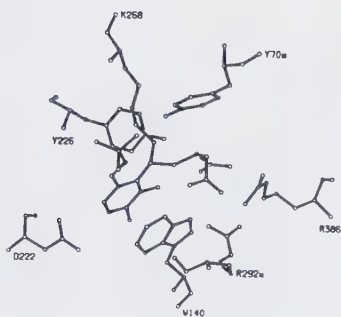
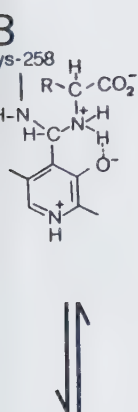
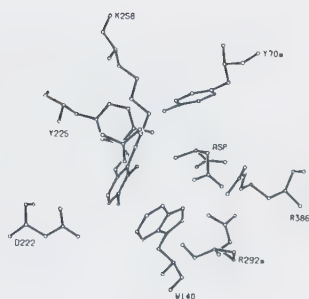
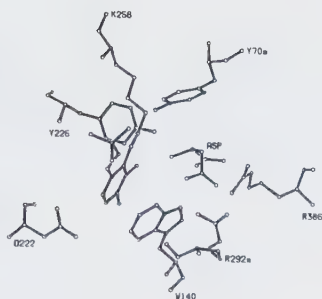
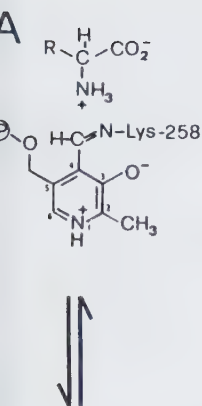
conformation equilibrium are observed in solution and in crystals. This conclusion is supported by the correlation of the conformational parameters of the enzyme in solution and the occurrence of the open and the closed form in crystals (for a more detailed discussion, see Pfister et al., 1985).

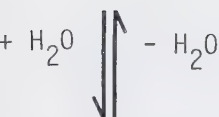
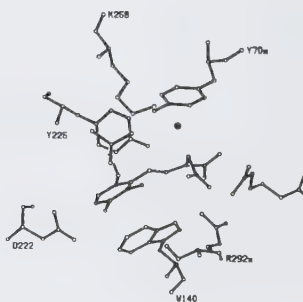
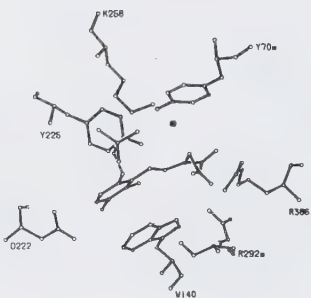
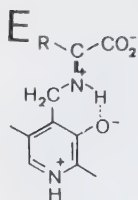
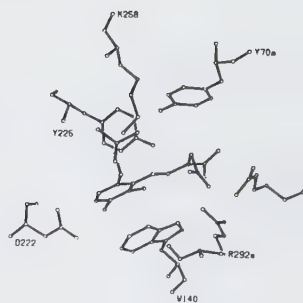
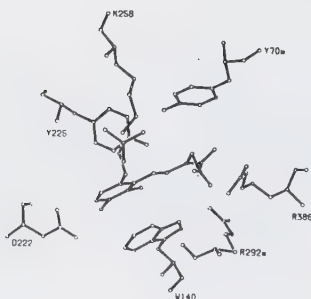
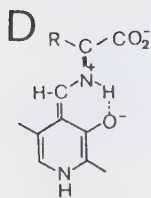
2. Mechanism of Action of Aspartate Aminotransferase

The following detailed hypothetical mechanism of enzymic transamination has been proposed by Kirsch et al. (1984). Below, this mechanism is described for the half-reaction of transamination starting from the PLP form of the enzyme plus aspartate and ending with the PMP form plus oxalacetate as product. The mechanism is largely based on crystallographic findings with mitochondrial aspartate aminotransferase. In view of the high degree of structural similarity between mitochondrial and cytosolic aspartate aminotransferase, mechanistic conclusions drawn for one isoenzyme may be expected to be valid also for the other one.

In the PLP enzyme, the coenzyme is anchored to the protein in the reactive bipolar ionic form (Ivanov and Karpeisky, 1969). The protonated N-1 is stabilized by a hydrogen bond and a salt bridge to Asp 222, below and behind it (Fig. 1, A). The deprotonated 3-hydroxyl group is involved in a strong hydrogen bond with Tyr 225.

Transamination - Aspartate, in cis-conformation, binds with its α - and its distal carboxylate group to the positively charged guanidinium groups of Arg 386 and Arg 292, respectively (Jenkins et al., 1959; Angielski and Rogulski, 1962; Khomutov et al.,





•water molecule

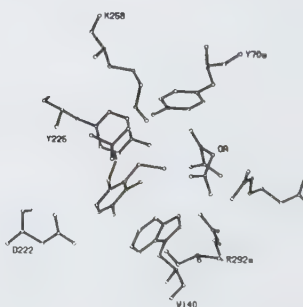
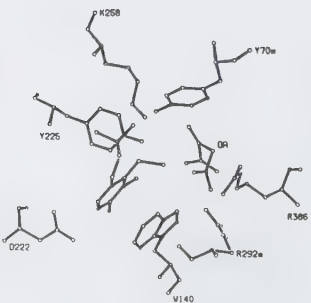
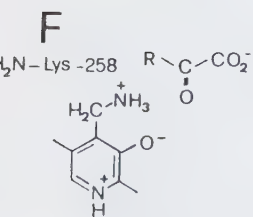


Fig. 1: Consecutive chemical steps of a half-reaction of enzymic transamination:



The stereo drawings show the active site of mitochondrial aspartate aminotransferase complexed with coenzyme and substrate at selected intermediate stages. It is based on the three-dimensional structures of mitochondrial aspartate aminotransferase in its unliganded forms and in a series of complexes with substrate analogs as well as on model-building experiments using computer graphics *.

The most important electrostatic interactions and hydrogen bonds between coenzyme, amino acid residues of the protein, and the substrate are shown.

- A Michaelis adsorption complex of PLP enzyme with aspartate, internal aldimine
- B Geminal diamine intermediate
- C External aldimine intermediate
- D Quinonoid intermediate
- E Ketimine intermediate
- F Michaelis adsorption complex of PMP enzyme with oxalacetate

1968; Michuda and Martinez-Carrion, 1970; Braunstein, 1973). A hydrogen bond between the α -amino group of the substrate and one of the two water molecules located near Tyr 70, is being established. The enzyme assumes the closed conformation, i.e. Arg 386, located in the small domain, comes as close as $\sim 3 \text{ \AA}$ to the coenzyme. Rotation of the coenzyme by an angle of $\sim 40^\circ$ around an axis through C2 - C5 (see also Ivanov and Karpeisky, 1969) facilitates the nucleophilic attack of the deprotonated substrate's amino group onto the coenzyme's C4' atom. The transimination to the external aldimine *via* the geminal diamine (Fig. 1, B and C) involves a rotation by $\sim 90^\circ$ of the two C-N-bonds along the C4-C4'-axis (Ivanov and Karpeisky, 1969; Furbish et al., 1969; Federiuk and Shafer, 1983). Subsequently, the uncharged ϵ -amino group of Lys 258 is split off. Lys 258 forms hydrogen bonds to Tyr 70 (-OH) and Gly 38 (-O). Its amino group lies within hydrogen bond distance to the substrate's C α and the coenzyme's C4' atom such that the proton transfer necessary for aldimine / ketimine tautomerization becomes possible.

* "Stereofigures from: J. N. Jansonius and M. G. Vincent (1987) Structural basis for catalysis by aspartate aminotransferase. In: F. Jurnak and A. McPherson (eds). Biological Macromolecules and Assemblies. Volume 3. New York."

Tautomerization - The resulting increase in pK of the aldimine nitrogen further facilitates deprotonation at C α to form the quinonoid intermediate (Fig. 1, D). The proton transfer from C α of glutamate to C4' of PMP as first reported by Gehring (1984) indicates that the 1,3-prototropic shift is a stepwise process catalyzed by a single acid/base group. Lys 258 has been suggested previously as a candidate for acid/base-catalyst (Snell, 1962; Braunstein, 1973; Fasella and Hammes, 1967). The topochemistry of the active site as determined by X-ray diffraction analysis indeed allows such a role of Lys 258. Protonation of C4' of the coenzyme at the pro-S position (Dunathan et al., 1968) produces the ketimine intermediate (Fig. 1, E). At the same time, the tilt angle of the coenzyme decreases (in the quinonoid intermediate $\sim 60^\circ$, in the ketimine intermediate $\sim 40^\circ$).

Hydrolysis - There may be two water molecules situated between Lys 258, Tyr 70, and Gly 38 (Kirsch et al., 1984). One of the water molecules, as a nucleophile, attacks the C4' atom of the coenzyme and transfers a proton to Lys 258. The tetrahedral intermediate (not shown) is cleaved to form PMP and oxo acid (Fig. 1, F). To date, few data on the Michaelis adsorption complex of the PMP form have been published.

3. Stereochemistry of Enzymic Transamination

During the aldimine/ketimine tautomerization of enzymic transamination a hydrogen is transferred from C α of the amino acid to C4' of the coenzyme (Fig. 1, C-E). In principle, addition of a proton at C4' atom, according to Hanson's (1966) convention, can take place on the si- or on the re-side. Ayling et al.

(1968) investigated the reaction catalyzed by pyridoxamine pyruvate transaminase. This enzyme catalyzes a half-reaction of the transamination cycle and uses nonphosphorylated PL and PM as substrates in sequence:



The authors reported an intramolecular transfer of 2 - 5% of deuterium from the alanine's C α to the coenzyme's C4'. When the same reaction was carried out in 99% D₂O with unlabeled substrates, the α -hydrogen of L-alanine was conserved by intramolecular transfer to the extent of 50%. Thus, a 10fold difference in intramolecular transfer is observed when proton transfer in D₂O is compared to deuterium transfer in H₂O. This evidence that the proton or deuterium which is transferred becomes equivalent to other deuteriums or protons, respectively, during the transfer indicates that the solvent itself takes part in the transfer.

With reference to the corresponding investigation with aspartate aminotransferase Dunathan (1971) wrote: "... it has not been possible so far to observe intramolecular proton transfer". Obviously, that was because exchange with the solvent was too fast. Gehring (1984) was the first to demonstrate intramolecular transfer of C α -tritium of glutamate to the coenzyme's C4'. He reported ~ 13% transfer in the case of mitochondrial aspartate aminotransferase using a fast mixing and quenching device in order to reduce the number of transamination cycles undergone by the enzyme and thus to minimize the accompanying exchange of label with water. The apoenzyme of aspartate aminotransferase catalyzes the reaction



with the nonphosphorylated cofactor acting as a dissociable substrate rather than as a prosthetic group (Wada and Snell, 1962).

There is a 500-fold decrease in the rate of the reaction of PM with 2-oxoglutarate in comparison to the rate of the transamination reaction catalyzed by the holoenzyme (Dunathan et al., 1968). PM labeled with deuterium in the R- and S-position of C4' loses one of the labels when subject to the apoenzyme-catalyzed reaction above. Dunathan (1966) was able to detect the release of deuterium as well as its appearance in the solvent. Conversely, incorporation of one deutron at C4' of PM was found when the reaction was carried out in D₂O as solvent (Dunathan et al., 1968). Tentatively, the product formed in the first and second case was identified as [C4'-D_{R or S}]PM, respectively. This assignment of the absolute configuration was confirmed by Besmer and Arigoni (1968). Thus, the exchange occurs only at the pro-S position at C4' of PM. This configuration corresponds to a protonation/deprotonation at C4' of the coenzyme-substrate adduct from the si-face of the coenzyme ring. Protonation/deprotonation at C4' from the si-face is common to many, i.e. to all of the following PLP-dependent enzymes (for a review, see Vederas and Floss, 1980): Alanine aminotransferase (substrate: alanine), Dunathan (1971); dialkyl aminotransferase (substrate: α -aminoisobutyrate), Bailey et al. (1970); glutamate decarboxylase (substrate: α -methylglutamate), Sukhareva et al. (1971); serine hydroxymethyl transferase (substrate: D-alanine), Voet et al. (1973); tryptophan synthetase (substrates: serine plus 2-mercaptoethanol), Dunathan and Voet (1974); Tsai et al. (1978); tryptophanase (substrate: serine), Vederas et al. (1978). The stereospecific proton release or proton addition is thought to be catalyzed by a specific acid/base group. In aspartate aminotransferase Lys 258, Tyr 70, or a water molecule that is situated at the active site (Kirsch et al., 1984; Gehring, 1984) have to be considered as possible catalysts (Fig. 1,E). In all forms of crystalline enzyme having Lys 258 not covalently linked to the coenzyme, a hydrogen bond is established between the ϵ -amino group and the phenolic hydroxyl group of Tyr 70. The

ϵ -amino group of Lys 258 is in a favorable position for proton transfer, the distances to C4' of the coenzyme and to C α of the substrate being 3.6 Å and 3.7 Å, respectively (distances were derived from X-ray analysis of the N-(5'-phosphopyridoxyl)-aspartate derivative of the enzyme; Kirsch et al., 1984). The phenolic oxygen of Tyr 70 which is slightly farther away ($\sim$ 4.6 Å) is less likely to be responsible for the proton transfer.

4. Aims of the Present Study

The present study investigates an apoenzyme-catalyzed model reaction of a dephosphorylated and thus dissociable cofactor with an aminotransferase substrate, i.e. the apoenzyme-catalyzed reaction of PL with aspartate, in order to assess the possible kinetic advantage of transaldimination *vs. de novo* formation of the external aldimine for the transamination reaction.

In the back reaction of enzymic transamination, the ketimine intermediate is deprotonated at C4' of the PMP moiety. The second part of this study examines whether the corresponding hydrogen in the pro-S position at C4' of enzyme-bound PMP is already labilized in the absence of substrate.

II. APOENZYME-CATALYZED MODEL REACTION FOR ASPARTATE AMINOTRANSFERASE

1. Introduction

In all vitamin B₆-dependent enzymes that catalyze reactions of amino acids PLP is bound covalently to an active site lysine residue. The first bond rearrangement step after binding of the amino acid substrate is thought to be the conversion of the so-called internal aldimine formed between PLP and Lys 258 into the external aldimine of PLP and the substrate amino acid *via* a geminal diamine intermediate (see Chapter I, Section 2). Studies with nonenzymic model systems indeed have shown transaldimination to occur *via* a geminal diamine intermediate rather than *via* the slower hydrolysis and subsequent *de novo* formation of the aldimine (Snell, 1962; Cordes and Jencks, 1962a, 1962b; Tobias and Kallen, 1975). This notion is generally held to apply also for the enzymic PLP-dependent reactions though to date it has not been possible to obtain clear evidence for or against either pathway. The present study investigates an enzymic model reaction, i.e. the apoenzyme-catalyzed reaction of PL with aspartate, in order to assess the possible kinetic advantage of transaldimination *vs.* *de novo* formation of the external aldimine for the overall transamination reaction.

The first apoenzyme-catalyzed reaction of a dephosphorylated and thus dissociable cofactor with an aminotransferase substrate, i.e. PL + aspartate $\rightleftharpoons$ PM + oxalacetate, was reported by Wada and Snell (1962). The reaction proved to be 3 orders of magnitude slower than the transamination reaction catalyzed by the holoenzyme with the phosphorylated non-dissociable cofactor. A continuous photometric assay of the reaction of PM and oxalacetate was established by Dunathan et al.

(1968). In the present work a photometric assay for the half-reaction $\text{PL} + \text{aspartate} \rightarrow \text{PM} + \text{oxalacetate}$ was established. In comparison to the proper enzymic transamination this reaction has the advantage that the half-reaction from PL to PM can be followed as a continuous turnover reaction in the absence of the reverse half-reaction. By choice of proper conditions, the reaction can be made to proceed either from the preformed internal aldimine or by *de novo* formation of the external aldimine; it thus has been used to compare the two pathways.

2. Experimental Procedure

2.1. Materials

Mitochondrial aspartate aminotransferase ($230 \text{ units mg}^{-1}$ at 25°C) was isolated from chicken heart as described previously (Gehring et al., 1977). Apoenzyme was prepared by dialysis of the PMP form of the enzyme (obtained by adding 2 mM cysteine sulfinate to the holoenzyme) against 0.5 M sodium phosphate, pH 5.2, at 4°C (Schlegel and Christen, 1974). Subsequently, the enzyme was transferred into 50 mM HEPES, pH 7.5 or 8.0, by gel filtration over Sephadex G-25. Apoenzyme thus prepared contained 1 mol of tightly bound inorganic phosphate (P_i) per mol subunit. P_i was determined according to Chen et al. (1956) in the supernatant after precipitating the protein with 12.5% (w/v) trichloroacetic acid.

L-Aspartic acid, L-glutamic acid, 2-oxoglutaric acid, sodium borohydride, and sodium hydrogen carbonate were from Fluka, HEPES was from Calbiochem. Pyridoxal hydrochloride, pyridoxal

5'-phosphoric acid, pyridoxaminedihydrochloride, oxalacetate, sodium dihydrogen phosphate monohydrate, and sodium sulfite were from Merck, sodium phosphite was from Riedel-de Haën and Sephadex G-25 from Pharmacia.

2.2. Determination of Enzymic Activity and Protein Concentration

Enzymic activity was measured in a coupled assay with malate dehydrogenase and 20 mM aspartate plus 20 mM 2-oxoglutarate as substrates (Birchmeier et al., 1973). Protein concentration was determined photometrically ($\epsilon_{280} = 7.0 \times 10^4 \text{ M}^{-1}$).

2.3. Preparation of P_i -free Mitochondrial Apoaspartate Aminotransferase

(a) Anion exchange HPLC (TSK DEAE-5PW column from LKB, $7.5 \times 75 \text{ mm}$, 50 mM HEPES, pH 7.5, 0.4 ml min^{-1}) of the apoenzyme (6 - 65 μmol ; prepared as described above) efficiently removes $\sim 95\%$ of bound P_i .

(b) Bound P_i can also be displaced by hydrogen carbonate. Apoenzyme containing 1 mol P_i per mol subunit was chromatographed on a column of Sephadex G-25 ($2 \times 44 \text{ cm}$, equilibrated and eluted with 50 mM HEPES plus 0.5 M hydrogen carbonate, pH 8.0), and lost $\sim 97\%$ of bound P_i .

(c) P_i -free apoenzyme is also obtained if the procedure as described by Schlegel and Christen (1974) is modified by using 0.5 M phosphite instead of P_i . The apoenzyme thus prepared contained 0.01 mol P_i per mol subunit. Its phosphite content was not measured. With respect to its interaction with PL it was indistinguishable from P_i -free apoenzyme prepared according methods (a) or (b).

2.4. Determination of Internal Aldimine by Reduction with Sodium Borohydride

After preincubation of apoenzyme with PL, sodium borohydride (final concentration 20 mM; 200 mM stock solution in 10 mM NaOH) was added. After chromatography on a column of Sephadex G-25 (4 x 160 mm; 50 mM HEPES, pH 7.5) and reconstitution with PLP (300 μ M, 20 min, room temperature), enzymic activity was determined. The loss in reconstitutable activity corresponds to the preformed aldimine linkage between apoenzyme and PL. In a control reduction in the absence of PL, $\sim$ 10% of reconstitutable activity were lost during the procedure. The indicated values are corrected for this unspecific decrease in reconstitutibility.

3. Results

3.1. Monitoring the Apoenzyme-catalyzed Transamination Half-Reaction of PL and Aspartate

The reaction $\text{PL} + \text{aspartate} \rightarrow \text{PM} + \text{oxalacetate}$ as catalyzed by mitochondrial aspartate aminotransferase from chicken was followed by measuring the increase in absorption at 336 nm due to the transformation of PL into PM. The wavelength of 336 nm was chosen because it corresponds with the isosbestic point of PL and both the internal aldimine of PL with apoenzyme and the aldimine of PL with aspartate (Fig. 2). The reaction (Fig. 3, curve A) was started by the addition of 40 mM aspartate to a mixture of PL and apoenzyme (containing 1 mol tightly bound P_i per mol subunit, see Iriarte et al., 1985, and Experimental Procedure).

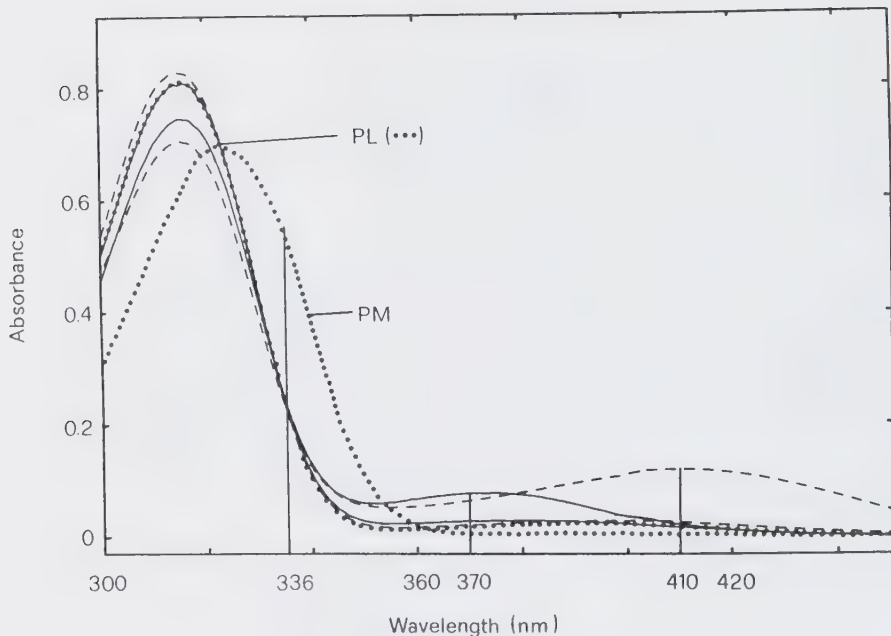


Fig. 2: Isosbestic point at 336 nm of PL, internal aldimine, and aldimine of PL with aspartate. Absorption spectrum of PL, 0.1 mM in 50 mM HEPES, pH 7.5, $\lambda_{\max}$ 318 nm, 25°C, (·····). Formation of the internal aldimine from P_i -free apoenzyme (11 μ M) and PL (0.1 mM) in the same buffer, immediately after mixing (the spectrum almost coinciding with that of PL) and after 5 min, $\lambda_{\max}$ 370 nm, (—). Formation of the aldimine from PL (0.1 mM) and aspartate (100 mM) in the same buffer, immediately after mixing and after 10 min, $\lambda_{\max}$ 410 nm, (---). For reference, the spectrum of PM (0.1 mM in the same buffer, $\lambda_{\max}$ 325 nm) is given (····). $\Delta\epsilon_{336}^{\text{PM-PL}}$ is 2.0 $\text{mM}^{-1} \text{cm}^{-1}$ or 3.2 $\text{mM}^{-1} \text{cm}^{-1}$ at pH 8.0 or pH 7.5, respectively.

The reaction of PM with oxalacetate as catalyzed by mitochondrial apoaspartate aminotransferase is accelerated by 50 mM sodium phosphate (Yagi et al., 1982). In contrast, an inhibitory effect of P_i has been reported in the case of the cytosolic isoenzyme (Wada and Morino, 1964). In the present study, the accelerating effect of phosphate (35-fold) on the mitochondrial apoenzyme could be confirmed. The reaction of PL

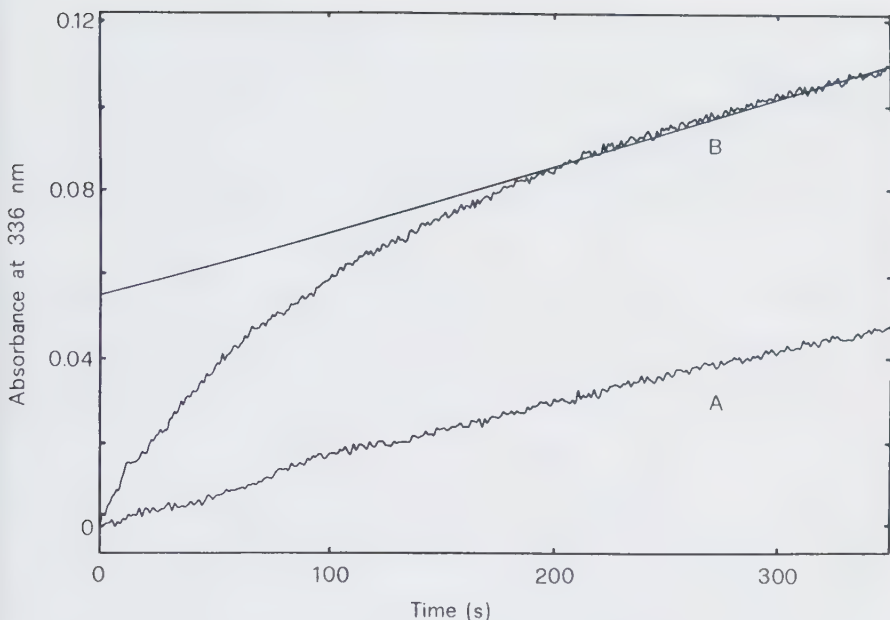


Fig. 3: The apoenzyme-catalyzed reaction $\text{PL} + \text{aspartate} \rightarrow \text{PM} + \text{oxalacetate}$, (A) without and (B) with preformation of internal aldime. In experiment A, the reaction mixture contained apoenzyme ($30 \mu\text{M}$) and $175 \mu\text{M}$ PL in 50 mM HEPES, pH 8.0, 25°C . After a preincubation of 5 min, the reaction was started by the addition of 0.5 mM aspartate. In experiment B, conditions were the same except the additional presence of 250 mM hydrogen carbonate. Extrapolation of the steady state phase to zero time gives the extent of transient product formation ($27.5 \mu\text{M}$ in the present experiment; obtained with $\Delta\epsilon_{336}^{\text{PM-PL}} = 2.0 \text{ mM}^{-1} \text{ cm}^{-1}$; see Fig. 2). The steady state rate corresponds to the rate observed in experiment A.

with aspartate as catalyzed by mitochondrial apoaspartate aminotransferase has been found to be slightly inhibited by P_i . A marked acceleration of the reaction is brought about by hydrogen carbonate (Table I). This effect was not observed for the reaction of PM with oxalacetate (see also Yagi et al., 1982).

TABLE I: Effect of anions (20 mM) on the initial rate of the reaction of PL (400 μ M) with aspartate (40 mM) as catalyzed by apoenzyme (22 μ M; containing 1 mol tightly bound inorganic P_i per mol in 50 mM HEPES, pH 7.5, 25°C). In all cases the reaction was started by the addition of aspartate. The effect of sulfite was not examined because it was found to react with PL. The strong inhibitory effect on the reverse reaction (PM plus oxalacetate) reported previously (Yagi et al., 1982) is probably due to reductive trapping of the formed PL by sulfite.

Anion	Relative initial rate
None	1.0 ^a
Phosphate ($H_2PO_4^-/HPO_4^{2-}$)	0.7
Phosphite ($H_2PO_3^-$)	1.0
Hypophosphite ($H_2PO_2^-$)	0.9
Arsenate ($H_2AsO_4^-/HAsO_4^{2-}$)	0.8
Sulfate	0.2
Chloride	1.2
Formiate	1.2
Hydrogen carbonate (HCO_3^-/CO_3^{2-})	3.2

^a Velocity 26 μ M min⁻¹

The faster initial rate measured in the presence of hydrogen carbonate is due to a faster transient phase of product formation. After preincubation of apoaspartate aminotransferase with PL for > 5 min in the presence of 250 mM hydrogen carbonate, the reaction after having been started by the addition of aspartate showed a distinct "burst" of PM formation (Fig. 3, curve B). In the absence of hydrogen carbonate no such "burst" is observed (Fig. 3, curve A). The occurrence of the "burst" correlates with the preformation of the internal aldimine (see Subsection 3.2.). In the absence of hydrogen carbonate only 0.05 mol aldimine per mol subunit could be detected by borohydride reduction (see Experimental Procedure) after incubation with up to 650 μ M PL. The apoenzyme used in this experiment was prepared by dialysis against phosphate (see Experimental Procedure) and its active site contained the tightly bound phosphate ion. Apparently, the bound phosphate ion prevents the formation of the internal aldimine. The dissociation constant of the mitochondrial isoenzyme for P_i is not exactly known; it has been reported to be somewhat higher (Iriarte et al., 1985) than that of the cytosolic isoenzyme ($K_d = 1.5 \mu$ M; Vergé et al., 1979). In the presence of hydrogen carbonate, the phosphate ion is displaced from the active site, gel filtration in the presence of hydrogen carbonate providing a convenient means to free apoenzyme from the tightly bound P_i (see Experimental Procedure). In contrast to the P_i -containing apoenzyme, P_i -free apoenzyme is capable of forming the internal aldimine.

3.2. Preformation of Internal Aldimine

In the reaction of apoenzyme with PL in the presence of hydrogen carbonate (Fig. 3, curve B), extrapolation of the slower steady state phase to zero time yields an increase in absorbance at 336 nm during the transient phase which corresponds to

the concentration of preformed internal aldimine: Coinciding saturation curves were obtained when the extent of the burst, the initial rate of the burst phase, or the internal aldimine as detected by reduction with borohydride were plotted as a function of the concentration of PL (Fig. 4). The absorption spectrum of the PL enzyme ($\lambda_{\max}$ 370 nm; Fig. 2) differs from that of general Schiff bases ($\lambda_{\max}$ 410 nm) including those with nonactive site lysine residues. The reaction of PL with nonactive site lysine residues was studied after reduction of the internal aldimine of PLP holoenzyme with sodium borohydride and removal of excess borohydride. The reaction of the reduced enzyme (130 μ M) with maximally 4.5 mM PL in 50 mM HEPES, pH 7.5, at 25°C was followed photometrically at 410 nm (see Fig. 2). The rate constants were $k'_{\text{on}} = 0.019 \text{ min}^{-1} \text{ mM}^{-1}$ and $k'_{\text{off}} = 0.164 \text{ min}^{-1}$ and are comparable to those found by Gilmer et al. (1977) for the reaction of nonactive site lysine residues of pyridoxamine pyruvate aminotransferase with 5'-deoxypyridoxal ($k'_{\text{on}} = 0.06 \text{ min}^{-1} \text{ mM}^{-1}$, $k'_{\text{off}} = 0.168 \text{ min}^{-1}$). The end value of ΔE_{410} indicated that 6.7 surface lysine residues per subunit had reacted with PL ($\Delta \epsilon_{410} = 3.75 \text{ mM}^{-1} \text{ cm}^{-1}$ per modified nonactive site lysine residue; Gilmer and Kirsch, 1977). The same number (n) was obtained from a graphical analysis according to the equation $K_d = ([\text{Enzyme}_0] - Z) ([\text{PL}_0] - nZ) \cdot Z^{-1}$, where $[\text{Enzyme}_0]$ is the total enzyme concentration, Z is $[\text{Enzyme}_0] \cdot (\Delta E_{\text{obs}} / \Delta E_{\text{max}})$, $[\text{PL}_0]$ the total concentration of PL (Ayling and Snell, 1968).

The rate constants k_{on} and k_{off} for the reversible second-order reaction of PL with P_i -free apoenzyme were determined as described in footnote (a) of Table II. The value for K_d as calculated from k_{on} and k_{off} (Scheme I) corresponds with the K_d value determined by photometric titration (Table II) and exceeds somewhat that determined previously for pyridoxamine pyruvate aminotransferase ($K_{d,\text{PL}} = 10 \text{ } \mu\text{M}$; Ayling and Snell, 1968).

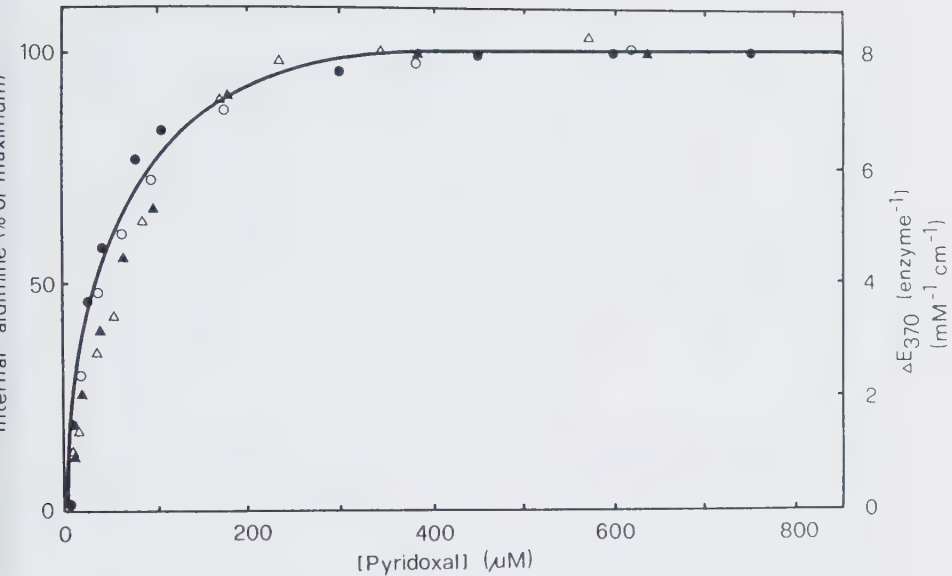


Fig. 4: Formation, detection, and reaction of internal aldimine in the presence of 250 mM hydrogen carbonate in 50 mM HEPES, pH 8.0, 25°C.

o Formation of internal aldimine followed photometrically at 370 nm (see Fig. 2). The equilibrium values reached 5 min after addition of pyridoxal at the indicated concentrations to apoaspartate aminotransferase (30 μM) are plotted.

● Internal aldimine detected by trapping with sodium borohydride (see Experimental Procedure). Conditions were the same as in the preceding experiment except the concentration of apoenzyme which was 26 μM. The maximum value reached was 1 mol internal aldimine per mol subunit. Together with the maximum ΔA_{370} measured in the experiment above (o), $\epsilon_{370} = 8.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for the internal aldimine was calculated.

▲ Relative initial rates of the reaction of PL and aspartate as catalyzed by apoenzyme. After preformation of the internal aldimine (28 μM) at increasing concentrations of PL (for other conditions, see Fig. 3, Experiment B), the reaction was started by the addition of 0.5 mM aspartate and the initial transient rate of PM formation was measured (maximum value at 0.5 mM aspartate = 1.57 min^{-1}).

Δ Extent of transient product formation as estimated by extrapolation of the presteady-state phase of Experiment B in Fig. 3 to zero time; $\Delta \epsilon_{336}$ was divided by $\Delta \epsilon_{336} = 2.0 \text{ mM}^{-1} \text{ cm}^{-1}$ (see Fig. 2). The maximum value corresponds with the subunit concentration of the apoenzyme.

Consistent with the notion that the effect of hydrogen carbonate is due to displacing the tightly bound phosphate ion from the active site, virtually the same kinetic and binding constants were measured in the case of apoenzyme that had been freed of tightly bound P_i by anion exchange HPLC, or that had been prepared with phosphite (0.5 M, pH 5.2) instead of P_i (Table II). Measurement of k_{obs} of internal aldimine formation at different concentrations of PL (see Table II, footnote (a) in the presence of increasing concentrations of hydrogen carbonate (5 - 250 mM in 50 mM HEPES, pH 8.0) shows that hydrogen carbonate ($K_d = 14$ mM) increases k_{on} but does not change k_{off} . This finding is again consistent with the conclusion that HCO_3^-/CO_3^{2-} facilitates the formation of the internal aldimine by displacing the tightly bound phosphate ion from the active site.

The formation of the internal aldimine as compared to corresponding nonenzymic reactions is quite fast. The reaction of PL (0.28 mM) with aspartate (10 - 100 mM in 50 mM HEPES, pH 7.5 or 8.0) could be followed photometrically at 410 nm. Plotting k'_{obs} of nonenzymic aldimine formation vs. the concentration of aspartate gave $k'_{on} = 0.00064 \text{ min}^{-1} \text{ mM}^{-1}$, $k'_{off} = 0.19 \text{ min}^{-1}$ at pH 7.5, and $k'_{on} = 0.0013 \text{ min}^{-1} \text{ mM}^{-1}$, $k'_{off} = 0.22 \text{ min}^{-1}$ at pH 8.0. The values are comparable with those reported for the reaction of PL and ϵ -aminocaproic acid (Wiesinger and Hinz, 1984) and correspond with the K_d of the PL-aspartate adduct (Metzler, 1957). Nonenzymic reactions of PLP are about one order of magnitude faster than those of PL because in the latter case the aldehyde group is not free but exists as the less reactive hemiacetal with the 5'-hydroxyl group (Heyl et al., 1951; Wiesinger and Hinz, 1984). For the reaction of PLP with ϵ -aminocaproic acid (Wiesinger and Hinz, 1984) and glutamic acid (Mackay, 1963) k'_{on} is $0.0071 \text{ min}^{-1} \text{ mM}^{-1}$ and $0.018 \text{ min}^{-1} \text{ mM}^{-1}$, respectively.

The nonenzymic reaction of free PL and aspartate at the concentrations used in the experiments with the enzyme (maximally 800 μ M PL and 40 mM aspartate, respectively) is thus at least 1 order of magnitude slower than the enzymic model reactions both *via* transaldimination and *de novo* formation of the external aldimine (see Scheme I, k_{cat} values). Hence, preformation of the external aldimine, i.e. formation of the PL-amino acid aldimine prior to binding to the apoenzyme, is a quantitatively insignificant pathway in the apoenzyme-catalyzed conversion of PL to PM.

3.3. Transaldimination vs. *de novo* Formation of External Aldimine

The kinetic parameters of the P_i -free apoenzyme for the total half-reaction of transamination were obtained from preincubation experiments with varying concentrations of PL and amino acid substrate (aspartate) (for conditions, see footnote (d) of Table II). The data were analyzed by secondary plots and found to fit an ordered bi bi system (Segel, 1975).

The K_m value for PL is given in Table II, the K_m value for aspartate as well as k_{cat} are given in the Scheme I (equation 1).

The kinetic parameters for the reaction of P_i -containing apoenzyme which shows no transient phase (Fig. 3, curve A) were determined in the same way and are also indicated in Scheme I (equation 2). Again, the data fit an ordered bi bi system. In view of the relatively slow reaction path *via* a preformed PL-Asp aldimine adduct (see Subsection 3.2.) we conclude that in the case of the P_i -containing apoenzyme, transamination occurs *via* the apo·PL complex and *de novo* formation of the external aldimine.

TABLE II: Formation of internal aldimine between PL and phosphate-free apoaspartate aminotransferase prepared by different methods: Rate constants of formation and decay, k_{on} and k_{off} , respectively, dissociation constant $K_{d,PL}$, and $K_{m,PL}$.

Preparation of P_i -free apoenzyme	k_{on} ^a ($\text{min}^{-1} \text{ mM}^{-1}$)	k_{off} ^a (min^{-1})	$K_{d,PL}$ ($k_{off} \cdot k_{on}^{-1}$) (μM)	$K_{d,PL}$ ^b (μM)	$K_{m,PL}$ ^c (μM)
Hydrogen carbonate ^e	5.9	0.32	54	56	64 (80 ^d)
HPLC ^f	6.7	0.32	48	65	46
Phosphite ^g	7.8	0.33	42	40	69

^a Apoenzyme (27 μM) was reacted with PL (0 - 650 μM) in 50 mM HEPES, pH 8.0, 25°C. The reaction was followed at 370 nm, the absorption maximum of the internal aldimine. The rate constants for the reversible second-order reaction were determined by plotting k_{obs} vs. the sum of the concentration of unliganded apoenzyme and free PL. The slope and the intercept on the k_{obs} axis give k_{on} and k_{off} , respectively (Gilmer et al., 1977).

^b The internal aldimine formed at equilibrium was measured photometrically ($\epsilon_{370} = 8.0 \text{ mM}^{-1} \text{ cm}^{-1}$; see Fig. 4 (●)). K_d was obtained from a double reciprocal plot of the data presented in Fig. 4 (o).

^c K_m was obtained from a double reciprocal plot of initial rate of the burst of the PL plus aspartate half-reaction of transamination (Fig. 4) as a function of the concentration of PL (5 - 550 μM). Conditions are described in the legend of Fig. 4.

^d $K_{m,PL}$ obtained from secondary plots for the PL plus aspartate half-reaction with varying concentrations of PL (25 - 650 μM) and aspartate (0.5 - 20 mM) in 50 mM HEPES, pH 8.0. After a preincubation (5 min) of apoenzyme (27 μM) with PL, the reaction was started by the addition of aspartate.

^e Apoenzyme was freed from tightly bound P_i by gel filtration in 250 mM hydrogen carbonate, pH 8.0 (see Experimental Procedure).

^f Apoenzyme freed from tightly bound P_i by anion exchange HPLC (see Experimental Procedure).

^g Apoenzyme prepared by dialysis against phosphite (0.5 M, pH 5.2) instead of P_i (see Experimental Procedure).

SCHEME I

Transaldimination



De novo formation of external aldimine



a Apoenzyme freed from tightly bound inorganic phosphate.

b Taken from Table II.

c Apoenzyme containing 1 mol of tightly bound inorganic phosphate per mol subunit.

d Aspartate (Conditions see footnote (d) of Table II).

e Oxalacetate.

A comparison of the transamination reactions as catalyzed on the one hand by P_i -containing and on the other by P_i -free apoenzyme shows that under the chosen conditions the two reactions in their steady state are equally fast (Fig. 3; see also k_{cat} values in the Scheme I). The k_{cat} values correspond to 0.13% of that of the holoenzyme for the overall transamination reaction with aspartate and 2-oxoglutarate as substrates ($k_{cat} = 10,350 \text{ min}^{-1}$, corresponding to 230 units mg^{-1}).

4. Discussion

The present study characterizes the single half-reaction of transamination undergone by PL with amino acid substrates and catalyzed by apoaspartate aminotransferase. It enlarges the previous observations of the effect of the tightly bound phosphate ion at the active site on the binding of the coenzyme and the formation of the internal aldimine (Vergé et al., 1979; Iriarte et al., 1985). The tightly bound P_i inhibits not only the binding of PLP and PMP (Busquets et al., 1985) but also of the unphosphorylated cofactor (see Scheme I). Conversely, PL at high concentration competes out the phosphate ion (Iriarte et al., 1985).

Coexistence of P_i and PL as internal aldimine is not possible, apparently because of steric hindrance due to the extra oxygen atom that remains between C5' and P when the phosphate ion is not linked to the 5'-hydroxyl group by an ester linkage. This interpretation is supported by the fact that formation of the internal aldimine becomes possible if phosphate is replaced by phosphite (either by preparing the apoform of the enzyme with phosphite instead of phosphate (see Experimental Pro-

cedure) or by freeing apoenzyme from P_i by HPLC and subsequently adding phosphite). In the presence of hydrogen carbonate (pH 7.5 or 8.0) the internal aldimine can also be formed, apparently because hydrogen carbonate displaces the tightly bound phosphate ion. Consistent with this view, apoenzyme may be freed completely from P_i by gel filtration in hydrogen carbonate (see Experimental Procedure).

Remarkably, the tightly bound phosphate ion does not appear to interfere with the covalency changes of the transamination reaction itself; the rates of the transamination reaction catalyzed by the P_i -free apoenzyme with preformed internal aldimine (reaction 1 in Scheme I) and the P_i -containing apoenzyme at saturating concentrations of PL (reaction 2 in Scheme I) are the same. The absence of interference with the covalency changes might relate to the syncatalytic repositioning of the coenzyme ring. The transition of the internal to the external aldimine is accompanied by a tilt of the coenzyme by an angle of $\sim 40^\circ$ around an axis through C2 - C5 removing C5' from the O atom of the phosphate ion and thus releasing the steric hindrance. The subsequent elementary steps of the transamination reaction result in an even more pronounced tilt of the coenzyme ring (Kirsch et al., 1984). The results with the P_i -free apoenzyme clearly demonstrate that the covalent linkage between the pyridine and the phosphate moiety of the cofactor is essential. Formation of the internal aldimine and occupancy of the P_i binding site, conditions that are approximated in the apoenzyme liganded with PL and phosphite, do not suffice as preconditions for efficient catalysis. In accordance with this notion, mitochondrial aspartate aminotransferase reconstituted with the 5-ethylphosphonate derivative of PLP possesses 10% of the activity of the native holoenzyme (Metzler and Fonda, 1985; H.P. Tobler, unpublished).

Nonenzymic model reactions have shown that the rate of transaldimination is faster than that of the *de novo* formation of an aldimine (Cordes and Jencks, 1962; Snell, 1962) and even exceeds the turnover number of enzymic transamination (Tobias and Kallen, 1975). In enzymic transamination, the existence of the internal aldimine between PLP and the active site lysine residue was thus thought to contribute to the rate acceleration by replacing the *de novo* formation of the aldimine bond to the substrate amino acid with a faster transaldimination step *via* a geminal diamine intermediate. The present study shows that for the enzymic model system under investigation, i.e. the apoenzyme-catalyzed reaction $\text{PL} + \text{aspartate} \rightarrow \text{PM} + \text{oxalacetate}$, this conclusion is valid at low concentrations of PL, the rate of transaldimination (equation 1 in the Scheme I; reaction starting from the preformed internal aldimine) clearly exceeding that of *de novo* formation of the external aldimine (equation 2 in the Scheme I). However, with increasing concentration of PL the rate of reaction 2 increases up to a maximum value. Apparently, at saturating concentrations of the cofactor, a step subsequent to the external aldimine becomes rate-limiting. The similarity of the values of k_{cat} of the two reaction pathways suggests that the same step also determines the rate of reaction 1.

Thus, the present study with an enzyme-catalyzed model reaction which is 3 orders of magnitude slower than the holoenzyme-catalyzed transamination reaction does not support but also not exclude the notion of a kinetic advantage brought about by the existence of the internal aldimine bond between PL and the enzyme protein. The main rate-determining step in enzymic transamination, and probably also in the present model reaction, is the aldimine-ketimine tautomerization rather than the formation of the external aldimine (Kirsch et al., 1984; Gehring, 1986). Remarkably, in the reverse half-reaction

starting from the PMP form of the enzyme and an oxo acid substrate, the *de novo* formation of the ketimine is fast enough not to be rate-limiting. Apparently, it is facilitated by some mechanism other than transamination, perhaps by deprotonation at C4' of PMP already consequent to the formation of the Michaelis adsorption complex with the substrate (see Chapter III, Section 4).

Is there an other possible explanation of the general occurrence of an internal aldimine in PLP-dependent enzymes besides the above kinetic argument? After all, the formation of the internal aldimine as an obligatory intermediate of the transamination cycle has to date eluded experimental demonstration. It is perhaps important in this context to consider that PLP in glycogen phosphorylase is also bound *via* a lysine aldimine bond though it is not undergoing a transaldimination step. Conceivably, in the evolution of PLP-dependent enzymes covalent binding of the cofactor preceded the development of a specific binding site operating with multiple noncovalent interactions.

III. STEREOSPECIFIC LABILIZATION OF THE C4'-PRO-S HYDROGEN OF PYRIDOXAMINE 5'-PHOSPHATE IN ASPARTATE AMINOTRANSFERASE

1. Introduction

The double displacement mechanism of enzymic transamination encompasses the following principal chemical events:



where E-PLP is the PLP form, i.e. the so-called internal aldimine of the enzyme, and E-PMP its PMP form (for a detailed discussion of the mechanism of action of aspartate aminotransferase, see Chapter I, Section 2). The aldimine-ketimine tautomerization is partly rate-limiting. It appears to be a stepwise process catalyzed by a single acid/base group; recently, transfer of a proton from C α of the substrate glutamate to the pro-S position at C4' of PMP could be demonstrated (Gehring, 1984).

In the back reaction of enzymic transamination, the ketimine intermediate is deprotonated at C4' of the PMP moiety. The question is thus raised whether the corresponding hydrogen of enzyme-bound PMP is already labilized in the absence of substrate. The experiments with tritiated PMP reported in this thesis show indeed that after binding of the cofactor to apo-aspartate aminotransferase its pro-S ^3H at C4' exchanges with solvent water. In contrast, unbound PMP does not show any measurable exchange.

Nonproductive substrates, substrate analogs and other ligands modulate the labilization of the pro-S hydrogen at C4' of enzyme-bound PMP. The relation of these modulations to shift in the open/closed conformational equilibrium of the active site crevice (see Kirsch et al., 1984, Pfister et al., 1985) were investigated.

2. Experimental Procedure

2.1. Materials

Mitochondrial aspartate aminotransferase (230 U mg⁻¹ at 25°C) was isolated from chicken heart as described previously (Gehring et al., 1977a). Enzymic activity was measured in a coupled assay with malate dehydrogenase and 20 mM aspartate plus 20 mM 2-oxoglutarate as substrates (Birchmeier et al., 1973). Apoenzyme was prepared by dialysis of the PMP form of the enzyme (obtained by adding 2 mM cysteine sulfinic acid to the holoenzyme) against 0.5 M sodium phosphate, pH 5.2, at 4°C (Schlegel and Christen, 1974). Subsequently, the enzyme was transferred into 50 mM HEPES, pH 7.5, or 50 mM sodium cacodylate, pH 7.5, by gel filtration over Sephadex G-25. Cytosolic aspartate aminotransferase from pig was prepared as described elsewhere (Pfister et al., 1985). Alkaline phosphatase from calf intestine, NADH, NAD⁺ and mitochondrial malate dehydrogenase from pig heart were from Boehringer; L-aspartic acid, 2-oxoglutaric acid, L-glutamic acid, sodium borohydride, 5,5'-dithiobis-(2-nitrobenzoate), N-ethylmaleimide, maleic acid, dehydroascorbic acid from Fluka, pyridoxal 5'-phosphoric acid, pyridoxal hydrochloride, pyridoxamine 5'-phosphoric acid, oxalacetic acid from Merck, HEPES and monobromotrimethylammoniumbimane (bimane) from Calbiochem, 2-methyl-D,L-aspartate,

sodium cyanoborohydride and cacodylic acid from Sigma, $[^3\text{H}]\text{NaBH}_4$ (11 Ci mmol^{-1}) from the Centre d'études nucléaires de Saclay. D,L-*erythro*-3-Hydroxyaspartic acid was prepared according to Kornguth and Ballach (1960) and Sato et al. (1957). $[^3\text{H}]\text{H}_2\text{O}$ (5 Ci ml^{-1}), L-[U- ^{14}C]aspartic acid (229 mCi mmol^{-1}), L-[U- ^{14}C]glutamic acid (280 mCi mmol^{-1}), L-[U- ^{14}C]-tyrosine (504 mCi mmol^{-1}), and L-[G- ^3H]glutamic acid (34 to 43 Ci mmol^{-1} labeled in α - and β -position) from Amersham. About 13% of the total radioactivity of $[^3\text{H}]$ glutamate was confined to C $_{\alpha}$ (Gehring, 1984).

2.2. Tritiation of PMP and PNP at C4'

The procedure previously described for the tritiation of unphosphorylated pyridoxamine (Dunathan et al., 1968; Chang et al., 1982) was performed by Medipro Laboratories, Teufen, Switzerland. $[4'\text{-}^3\text{H}]\text{PMP}$ was prepared by reacting PMP (110 μmol) with PLP (38 μmol) in 1 ml $[^3\text{H}]\text{H}_2\text{O}$ (5 Ci ml^{-1}) at pH 4 and room temperature for 4 days in the dark. The reaction mixture was lyophilized and redissolved twice in 1 ml unlabeled water. For storage, the material was dissolved in 1.2 ml water and kept at -80°C . A sample corresponding to 1/6 of the total material was chromatographed on a DEAE-cellulose column (DE-32 from Whatman, 27 x 1.3 cm), equilibrated and eluted with 0.3 M pyridine/0.3 M acetic acid (Gehring, 1984). The PMP containing fractions were pooled, lyophilized, redissolved in 1 ml water, and stored before use at -20°C . The concentration of PMP was determined spectrophotometrically ($\epsilon_{293} = 9.0 \text{ mM}^{-1} \text{ cm}^{-1}$ in 0.1 M HCl; Peterson and Sober, 1954). Radioactivity was measured by scintillation counting. The specific radioactivity of tritiated PMP was $4.6 \times 10^6 \text{ cpm } \mu\text{mol}^{-1}$.

Preparation of [C4'-³H]PNP - [³H]PNP was prepared from PLP by reduction with tritiated sodium borohydride. PLP (10 μmol in 900 μl H₂O) was reacted with 36 nmol [³H]NaBH₄ (11 mCi μmol⁻¹, dissolved in 50 mM NaOH). After 10 min, unlabeled NaBH₄ (62.5 μmol, dissolved in 5 mM NaOH) was added. After further 5 min the hydrolysis of borohydride that had not been consumed was accelerated by acidification with 250 μmol HCl. The reaction mixture was then 3 times lyophilized and redissolved in 300 μl water. The concentration of PNP was determined photometrically ($\epsilon_{310} = 7.3 \text{ mM}^{-1} \text{ cm}^{-1}$ in 0.1 M NaOH; Peterson and Sober, 1954). The specific radioactivity of [³H]_{R,S}PNP was $45 \times 10^6 \text{ cpm } \mu\text{mol}^{-1}$.

2.3. Determination of ³H in Pro-S Position at C4' of [³H]PMP

[³H]PMP (0.2 μmol) was added to the same amount of unlabeled PMP in 50 mM HEPES (pH8.5, final volume 0.5 ml). Alkaline phosphatase (20 U) was added, and after 30 min at 25°C removed by centrifugation-assisted ultrafiltration (Centriflo CF 25, Amicon). High voltage electrophoresis of the ultrafiltrate on Whatman-3MM paper at pH 1.9 (acetic acid/formic acid/water 1:10:89) gave a single u.v. fluorescent spot that stained orange with ninhydrin (Gehring, 1977b). The dephosphorylation procedure did not change the specific radioactivity of the cofactor; no exchange of tritium into the water was observed. Subsequently, the pro-S ³H at C4' of [³H]pyridoxamine was determined by a procedure reported earlier (Dunathan et al., 1968) under the following conditions: [³H]Pyridoxamine (79 nmol) was added to cytosolic apoaspartate aminotransferase from pig (1 mg ml⁻¹) in 0.4 mM 2-oxoglutarate, 3 mM glutamate, 0.1 M Tris chloride, and 0.1 M sodium carbonate (pH 8.5, final volume 0.5 ml). In order to follow the time course of detritiation, samples were with-

drawn from the reaction mixture, and the reaction stopped by freezing in liquid nitrogen. The sum of sublimated and residual radioactivity corresponded with the total initial radioactivity. Maximum radioactivity released was 1.8×10^6 cpm μmol^{-1} which corresponds to 39% of the total radioactivity of [^3H]PMP.

2.4 High Performance Anion Exchange Chromatography of [^3H]PMP and Apoaspartate Aminotransferase

The apoform of mitochondrial aspartate aminotransferase and [^3H]PMP were efficiently freed from contaminating oxo acid and amino acid substrates by high performance anion exchange chromatography (TSK DEAE-5PW from LKB, 7.5 x 75 mm). The apoenzyme (0.2 - 3 mg) was chromatographed with 5 or 50 mM HEPES or 50 mM sodium cacodylate, each pH 7.5 and 0.4 ml min^{-1} ; [^3H]PMP (7 - 320 nmol, 4.6×10^6 cpm μmol^{-1}) was chromatographed with 50 mM pyridine/50 mM acetic acid (0.5 ml min^{-1}). Both apoenzyme and coenzyme were eluted after 3.0 - 3.5 ml. The coenzyme containing fractions were lyophilized and dissolved in water. The efficiency of separation was checked by adding ^{14}C -labeled amino acids (aspartate, glutamate, and tyrosine) and oxo acids (2-oxoglutarate, and oxalacetate) to the enzyme or coenzyme prior to the chromatography.

[^{14}C]Oxalacetate was synthesized from [^{14}C]aspartate (4.8×10^{-4} μmol , 438×10^6 cpm μmol^{-1}), unlabeled aspartate (1.4×10^{-4} μmol) and oxalacetate (0.25×10^{-4} μmol) in the presence of aspartate aminotransferase (8 μg) in a final volume of 150 μl (50 mM HEPES, pH 7.5, 3 h). Similarly, [^{14}C]oxoglutarate was produced from [^{14}C]glutamate (4×10^{-6} μmol , 546×10^6 cpm μmol^{-1}) and unlabeled 2-oxoglutarate (1.25×10^{-3} μmol) in a final volume of 250 μl (3 h). Samples containing pairs of labeled reaction products were chromatographed as

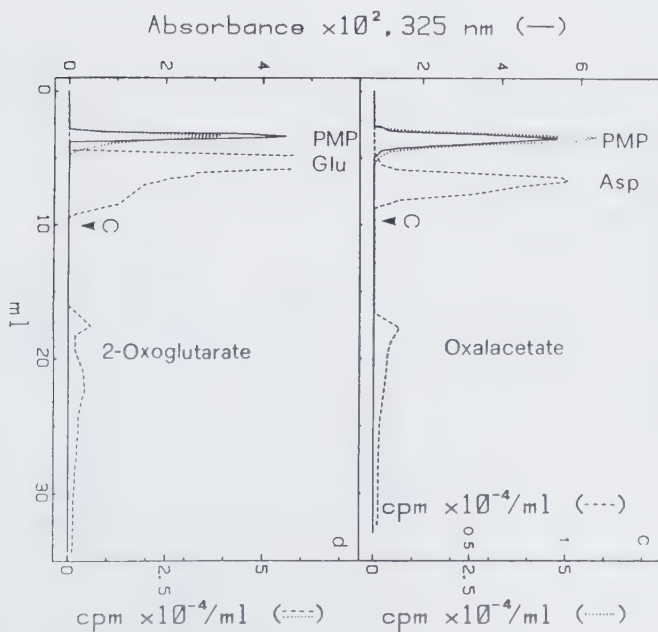
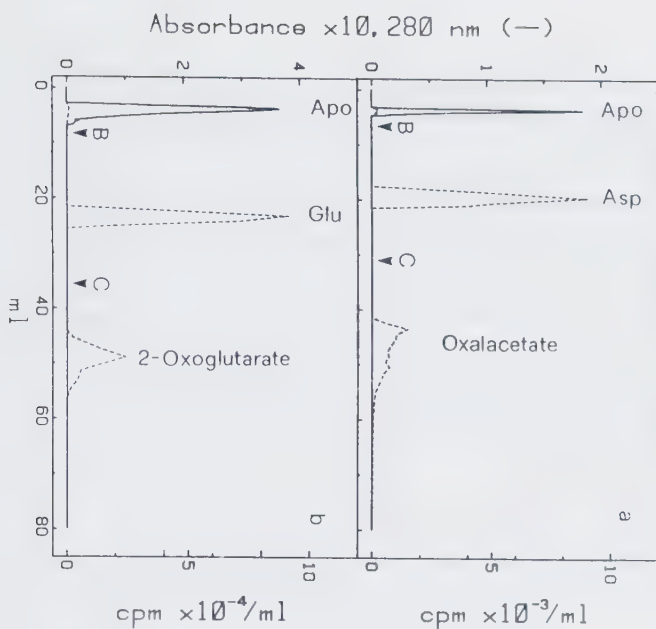


Fig. 5: High performance anion exchange chromatography of [^3H]PMP and apoaspartate aminotransferase - (a,b). Separation of the components of a mixture containing apoenzyme (0.12 mg) together with (a) [^{14}C]aspartate plus [^{14}C]oxalacetate or together with (b) [^{14}C]glutamate plus [^{14}C]2-oxoglutarate (for details, see text). Before being loaded with samples, the column was equilibrated with HEPES (50 mM, pH 7.5) which also served as elution buffer for the apoenzyme. [^{14}C]-Amino acids (---) were eluted with buffer B (50 mM pyridine/50 mM acetic acid, [^{14}C]oxo acids (---) with buffer C (0.1 M formic acid/0.1 M sodium sulfate). (c,d) Separation of the components of a mixture containing [^3H]PMP (..., 4.6×10^6 cpm μmol^{-1}) together with (c) [^{14}C]aspartate plus [^{14}C]oxalacetate, or together with (d) [^{14}C]glutamate plus [^{14}C]2-oxoglutarate. The sample was loaded onto the column equilibrated with buffer B (50 mM pyridine/50 mM acetic acid). This buffer system allowed elution of coenzyme and amino acids, well separated from each other, whereas, for elution of [^{14}C]oxo acids, buffer C (0.1 M formic acid/0.1 M sodium sulfate) had to be used.

described in Fig.5: [^{14}C]Aspartate plus [^{14}C]oxalacetate (total amount 1.8×10^{-4} μmol ; total specific radioactivity 320×10^6 cpm μmol^{-1}), or [^{14}C]glutamate plus [^{14}C]2-oxoglutarate (8.6×10^{-4} μmol , 410×10^6 cpm μmol^{-1}), or [^{14}C]tyrosine (5×10^{-4} μmol , 970×10^6 cpm μmol^{-1}) together with apoenzyme or [^3H]PMP.

The purification factors obtained for apoenzyme and coenzyme, respectively, were 2500 and 3500 in the case of the aspartate plus oxalacetate mixture, 5700 and 15,500 with glutamate plus 2-oxoglutarate, and 10,000 with tyrosine. The latter compound was chromatographed only with apoenzyme.

The amount of inorganic phosphate that is tightly bound to the active site of aspartate aminotransferase was efficiently decreased from 1 mol/mol subunit to < 0.1 mol/mol by high performance anion exchange chromatography (see Chapter II, Subsections 2.1. and 2.3.).

3. Results

3.1. ^3H Release from [^3H]Pyridoxamine 5'-Phosphate upon Binding to Apoaspartate Aminotransferase

Apoaspartate aminotransferase which has been freed of tightly bound inorganic phosphate (see Iriarte et al., 1985) by HPLC (see above) is rapidly reconstituted by [^3H]PMP in a phosphate-free buffer ($t_{1/2} < 1$ min; Fig. 6). Simultaneously, the release of ^3H is initiated as reflected in a continuous increase in sublimable radioactivity (Fig. 6). The release of ^3H follows first-order kinetics with $k = 5 \times 10^{-4} \text{ s}^{-1}$; $\text{SD} \pm 0.5 \times 10^{-4} \text{ s}^{-1}$, $n = 8$. The release of the ^3H catalyzed by the apoenzyme is 32,000 times faster than the overall random release of ^3H from [^3H]PMP (60 μM) in the absence of apoenzyme followed during 11 days under otherwise comparable conditions.

The final maximum amount of radioactivity released from enzyme-bound PMP corresponds to that released from the dephosphorylated coenzyme on incubation with apoaspartate aminotransferase (cytosolic isoenzyme from pig) plus oxo and amino acid substrate (see Experimental Procedure), a process which has been shown previously (Dunathan et al., 1968) to release stereospecifically the pro-S hydrogen from C4' of pyridoxamine. The following experiment confirmed that the proton that is released into water after reconstitution of mitochondrial aspartate aminotransferase corresponds indeed to the pro-S hydrogen at C4' of PMP, i.e. to the same hydrogen that is involved in the 1,3-prototropic shift from the ketimine to the aldimine intermediate during the enzymic transamination reaction (Dunathan et al., 1968). [^3H]PMP (173 nmol) was detritiated with mitochondrial apoaspartate aminotransferase (252 nmol in 2 ml) under the conditions of Fig. 1. After 230 min the reaction was quenched with 200 mM HCl. Subsequently, the pH value was adjusted to 8.5, the precipitate removed by

centrifugation, the coenzyme in the supernatant dephosphorylated as described under Experimental Procedure, and then twice lyophilized. Determination of [^3H] in the pro-S position at C4' with cytosolic apoaspartate aminotransferase showed that it had been decreased to 6% of its initial value during the exchange experiment.

3.2. Control Experiments Excluding a Possible Contamination with Substrates

In the present study it is of utmost importance to exclude the occurrence of transamination reactions between the PMP form of the enzyme and possibly contaminating oxo acid substrates. Routinely, both apoenzyme and [^3H]PMP were purified by high performance anion exchange chromatography (see Experimental Procedure). The following control experiments were performed: (1) Apoenzyme and [^3H]PMP were rechromatographed on the high performance anion exchange column. The rates of both reconstitution and ^3H exchange remained the same as after the first chromatography. (2) Reconstitution of apoenzyme in the presence of a fivefold higher concentration of [^3H]PMP (195 μM) or a threefold increase in the concentration of the apoenzyme at a constant concentration of [^3H]PMP resulted in the same rate and extent of ^3H release. This control experiment in addition indicates that only enzyme-bound PMP is detritiated. (3) Both apoenzyme (7.8 nmol) and [^3H]PMP (7.7 nmol) were treated with sodium borohydride (10 mM in a final volume of 200 μl) in order to reduce possibly contaminating oxo acids. The incubation was continued at 4°C for 24 - 72 h. After that time all borohydride had been hydrolyzed. Alternatively, borohydride was trapped after 20 - 25 min by the addition of 15 mM pyridoxal, 20 mM dehydroascorbic acid, or 15 mM NAD^+ . All these pretreatments did not alter the rate of ^3H exchange. The potential efficiency of the reduction of oxo acids with sodium borohydride was checked with 2-oxoglutarate. 2-Oxoglutarate (250 μM

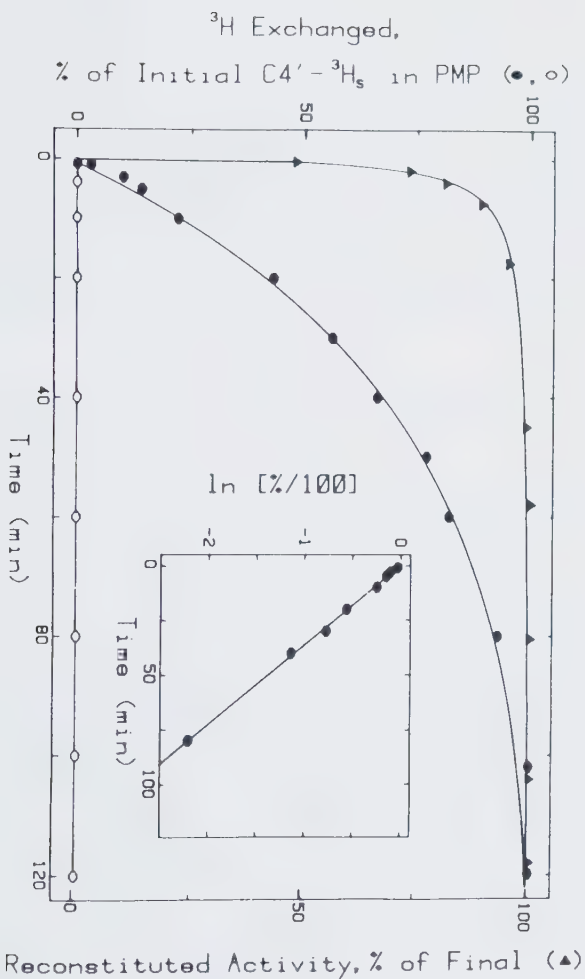


Fig. 6: Release of tritium from [^3H]PMP upon binding to apoaspartate aminotransferase. [^3H]PMP (38 μM , total radioactivity 4.6×10^6 cpm μmol^{-1}) was added to apoenzyme in equimolar subunit concentration (final volume 300 μl , 50 mM HEPES, pH 7.5, 25°C). The binding of the coenzyme was followed by removing samples at the indicated times and assaying them for aspartate aminotransferase activity ($\blacktriangle$). The final specific activity corresponded with that of holoenzyme prior to resolution (~ 230 units mg^{-1}). For following the detritiation of [^3H]PMP, samples (20 μl) were quenched in 100 μl 0.5 M HCl, immediately frozen in liquid nitrogen, and lyophilized for the determination of sublimable radioactivity ($\bullet$). The final maximum amount of sublimable radioactivity was 1.9×10^6 cpm μmol^{-1} . As a control, [^3H]PMP was added in the same concentration to the same buffer without apoenzyme and the sublimable radioactivity counted (o). In all samples, the sum of sublimable and residual radioactivity corresponded to the total radioactivity of [^3H]PMP added initially. In the inset, the exchange data are replotted semilogarithmically.

in 50 mM HEPES, pH 7.5) was reduced with 10 mM sodium borohydride for 6.5 min. On addition of the reduced substrate (15 μM) to the PMP form of aspartate aminotransferase (31 μM) in the presence of 10 mM sodium borohydride the activity of the enzyme remained unchanged. Apparently, 2-oxoglutarate had been completely reduced preventing the conversion of the enzyme to the borohydride-susceptible PLP form. In contrast, the enzymic activity was decreased to 52% of its initial value by the above treatment if the same amount of unreduced substrate was added to the PMP enzyme. (4) In order to check for possible contamination of the buffer substances with substrates, the rate of ^3H release was determined at varying buffer ion concentrations (Fig. 7). Apoenzyme (purified in 50 mM sodium cacodylate, pH 7.5) was reconstituted in varying concentrations of HEPES or sodium cacodylate. Both the rate of reconstitution with PMP, and the transaminase activity as measured at low substrate concentrations (60 μM aspartate and 110 μM 2-oxoglutarate) and thus reflecting an apparent $k_{\text{cat}}/K_{\text{m}}^{-1}$ value were not influenced by the type and the concentration of the buffer. In contrast, ^3H release in HEPES was twice as fast as that in cacodylate and in both buffers a maximum value was measured at a 50 mM concentration (Fig. 7).

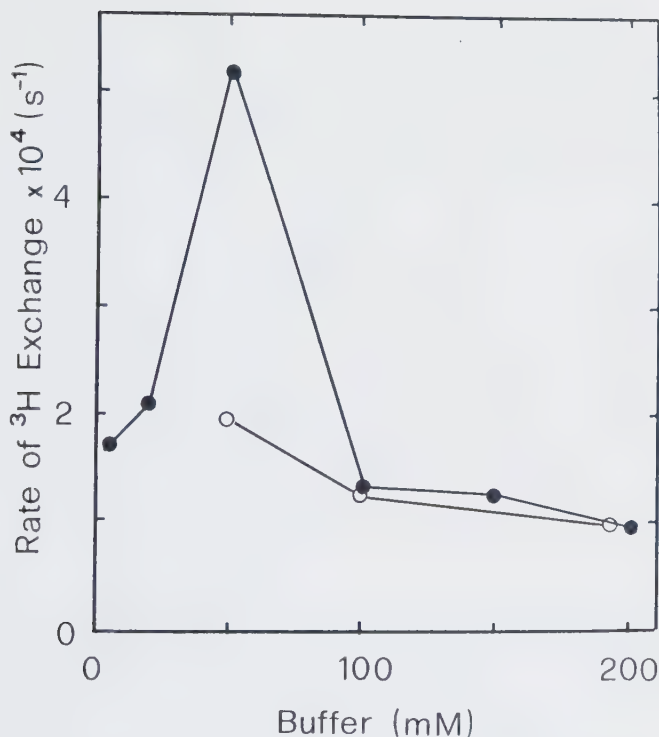


Fig. 7: Apoaspartate aminotransferase catalyzed detritiation of [³H]PMP, effect of buffer ion concentration. Apoenzyme was reconstituted with [³H]PMP at the indicated concentrations of HEPES, pH 7.5 (●), or sodium cacodylate, pH 7.5 (○). Other conditions were as for the experiment shown in Fig. 6. The rate of reconstitution was independent of buffer concentration. The rate of ³H release was determined as detailed in Fig. 6.

- (5) The distilled water was excluded as a source of contaminating oxo substrates by adding water (half of total volume) that had undergone flash evaporation to 1/200 of its initial volume to the reaction mixture. ³H was released at unchanged rate.
- (6) The pH-dependence of the rate of ³H release differs from that of the rate of the enzyme-catalyzed transamination reaction at low substrate concentrations (Fig. 8). This dis-

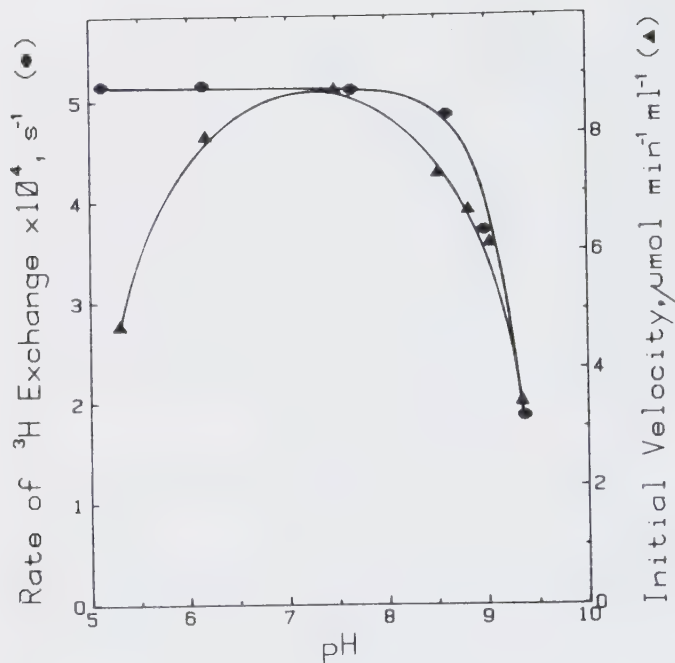


Fig. 8: pH-Dependence of ^3H release from enzyme-bound [^3H]PMP and of transaminase activity. Apoaspartate aminotransferase (subunit concentration 44 μM) was reconstituted with equimolar [^3H]PMP in 5 mM HEPES, pH 7.5. After attaining maximum activity, samples of enzyme (final subunit concentration 30 μM) were added to 25 mM sodium cacodylate, 25 mM sodium borate, pH as indicated, 25°C. The first-order rates of ^3H release ($\bullet$) were determined as described in Fig. 6. The initial velocities of the transamination reaction ($\blacktriangle$) were measured in the same buffers at substrate concentrations (60 μM aspartate and 110 μM 2-oxoglutarate) corresponding to 1/10 of the K_m values (Shrawder et al., 1972), and thus reflect apparent $k_{\text{cat}} K_m^{-1}$ values.

crepancy further indicates that the detritiation of PMP is not due to transamination reactions with contaminating substrates.

3.3. Attempt to Detect Apoenzyme-catalyzed Release of $C\alpha$ - 3H from [3H]Glutamate

Apoenzyme prepared as described under Experimental Procedure shows a residual transaminase activity of < 1% of that of the reconstituted enzyme. In order to eliminate the residual activity, apoenzyme (225 μM) was treated with 2-oxoglutarate (1 mM) and sodium borohydride (first addition 20 mM, second addition 1 mM). Apoenzyme treated in this way did no more show any activity but could be completely reconstituted (200 units mg^{-1}). After addition of [3H]glutamate (63×10^6 cpm μmol^{-1} , 11 mM) to apoenzyme (200 μM), the sublimable radioactivity was measured during a period of up to 5.5 h. No release of 3H from $C\alpha$ of the amino acid was detected.

3.4. Failure of Enzyme-bound [3H -C4' $_{R,S}$]Pyridoxine 5'-Phosphate to Release 3H

The contribution of the 4'-amino group of PMP was assessed by substituting this amino group with a hydroxyl group, i.e., by measuring the tritium release from enzyme-bound [3H -C4' $_{R,S}$]PNP (45×10^6 cpm μmol^{-1} , prepared as described under Experimental Procedure). The binding of the coenzyme derivative (380 μM) to the apoenzyme (38 μM) was followed by measuring the decrease in activity that could be reconstituted by unlabeled PMP (38 μM). Enzyme-bound [3H]PNP released no sublimable radioactivity within 16 h even when the apoenzyme had not been fully occupied by the coenzyme derivative. The addition of 20 mM aspartate and then 20 mM 2-oxoglutarate to the PNP enzyme was without effect. Apparently, the C4' amino group is essential for the labilization of the C4' pro-S hydrogen. The replacement of N4' by an oxygen atom might interfere with the required active site geometry, or prevent the electron withdrawing effect of the N4'-N ϵ Lys 258 hydrogen bond system.

3.5. Aspartate and Glutamate as Activators of the Labilization of the pro-S Hydrogen at C4' of [³H]PMP

Formation of the unproductive adsorption complex of the [³H]PMP enzyme with aspartate^a or glutamate results in a marked acceleration of ³H release (Fig. 9, Table III). The accelerating effect of these ligands on the first-order rate constant of ³H release (corrected for the release in the absence of the ligand) follows saturation kinetics (Fig. 11). Nonenzyme-bound [³H]PMP (60 μM) in the same buffer both in the presence of aspartate (6.25 mM) and in its absence releases < 1% of its total radioactivity within 16 h. Under saturating concentrations of aspartate, the enzyme-catalyzed release of ³H ($k = 0.21 \text{ s}^{-1}$) is 13×10^6 times faster than that of nonenzyme-bound [³H]PMP in the same buffer (Fig. 6). Taking into account a tritium isotope effect of 10 to 20, the rate constant of deprotonation of enzyme bound PMP is only 2 orders of magnitude slower than the molecular activity of aspartate aminotransferase for the overall transamination reaction ($k_{\text{cat}} = 172 \text{ s}^{-1}$).

3.6. The Unproductive Adsorption Complex of the Pyridoxamine Form of Aspartate Aminotransferase with Aspartate

In order to monitor the binding of aspartate to the PMP form of the enzyme and the concomitant conformational changes, aspartate aminotransferase that had been labeled (Sandmeier and Christen, 1984) with the fluorophor bimeane (monobromotrimethylammoniumbimeane; Kosower et al., 1979) was titrated fluorometrically with aspartate. Addition of aspartate to the PMP form of the enzyme results in a decrease in the fluorescence intensity by maximally 32% of the value of the unliganded holo-

^a All substrates and other ligands of the enzyme were treated with sodium borohydride (10 mM final concentration, pH 7.5, room temperature, for 24 - 72 h until borohydride had been completely hydrolyzed) in order to reduce completely any possibly contaminating oxo acid substrates.

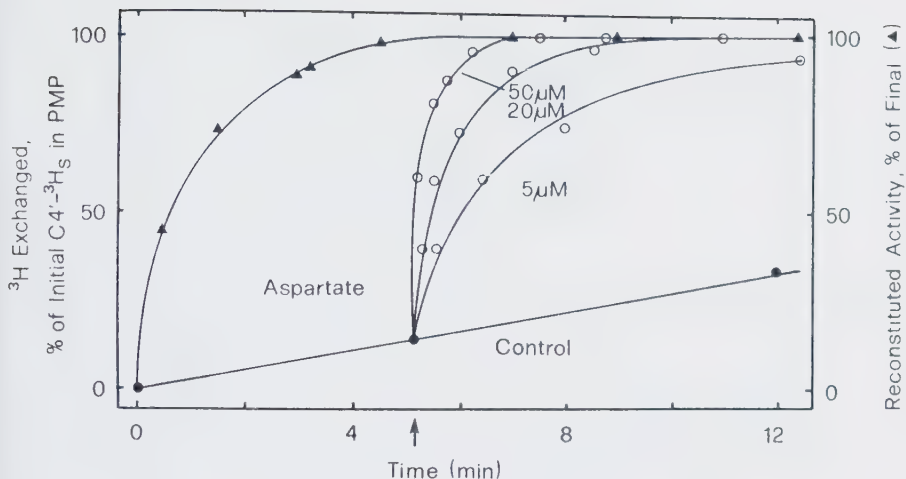


Fig. 9: Release of ^3H from $[^3\text{H}]\text{PMP}$ after binding to apoaspartate aminotransferase, acceleration by aspartate. Apoaspartate aminotransferase ($32\ \mu\text{M}$ in $50\ \text{mM}$ HEPES, $\text{pH } 7.5$) was reconstituted with a threefold molar excess of $[^3\text{H}]\text{PMP}$. The binding of the coenzyme was followed by removing samples from the reaction mixture and assaying them for aspartate aminotransferase activity ($\blacktriangle$). The final specific activity corresponded with that of holoenzyme prior to resolution ($\sim 230\ \text{units mg}^{-1}$). After completion of the reconstitution, samples were removed at the indicated times and the sublimable radioactivity was determined ($\bullet, \circ$). The addition of aspartate (arrow on the time axis) at the indicated concentrations accelerates ^3H release as compared to the control without aspartate. The enzymic activity is not changed by the addition of aspartate.

enzyme (Fig. 10). The decrease in fluorescence intensity reflects a change in conformation of the protein (Sandmeier and Christen, 1984) corresponding to the ligand-induced and syn-catalytic changes in conformation that have been detected previously by differential chemical and proteolytic modification (Gehring and Christen, 1978; Sandmeier and Christen, 1980), peptide hydrogen exchange (Pfister et al., 1978), and X-ray crystallography (Kirsch et al., 1984). The decrease in fluorescence intensity on titration of the PMP enzyme with aspartate (up to $4.5\ \text{mM}$) yields a saturation curve ($K_d = 440\ \mu\text{M}$)

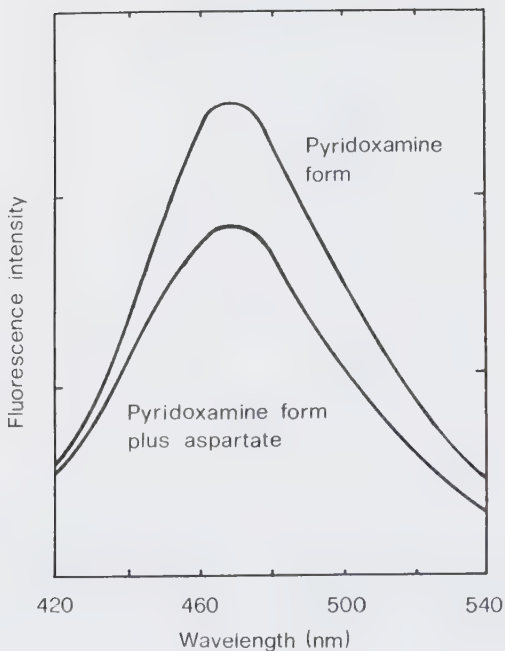


Fig. 10: Effect of aspartate (6 mM) on the fluorescence intensity of the PMP form of bimane-labeled aspartate aminotransferase (7 μ M in 50 mM HEPES, pH 7.5, 25°C). Excitation was at 380 nm.

coinciding with that describing the first order rate constant of ^3H release as a function of aspartate concentration ($K_d = 400 \mu\text{M}$; Fig. 11). Glutamate (up to 150 mM) did not induce a decrease in fluorescence intensity.

X-Ray crystallographic analysis of the unproductive complex of the PMP form of the enzyme with aspartate showed that phosphate ions might bind to the same site where aspartate is bound (Eichele, 1980). Consistent with this observation, the PMP form binds aspartate rather tightly ($K_d = 440 \mu\text{M}$) in the absence of

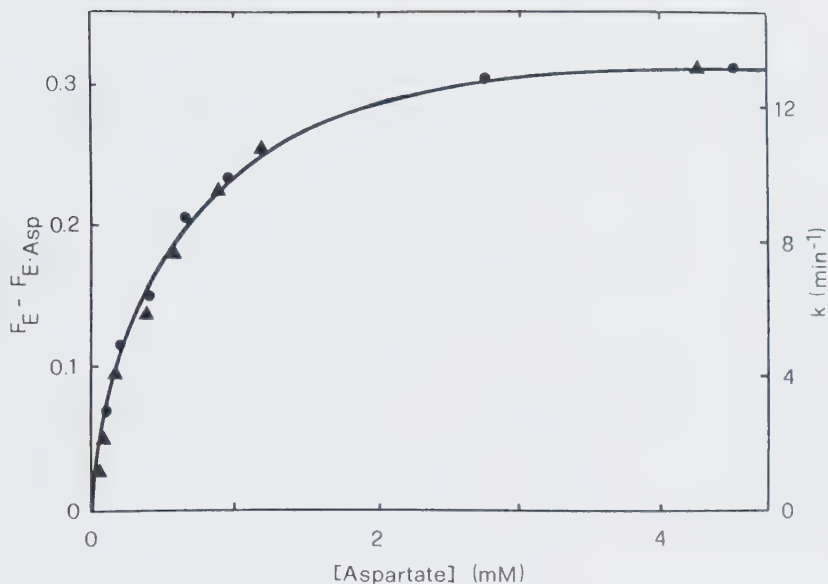


Fig. 11: Saturation curve obtained by measuring the accelerating effect on ^3H release and the effect on the fluorescence properties of bimane-labeled enzyme as a function of the concentration of aspartate. The first order rates of ^3H release (●) were obtained from experiments as in Fig. 6 and corrected for the rate observed in the absence of aspartate. The addition of aspartate at the indicated concentrations to the PMP form of aspartate aminotransferase (7 μM in 50 mM HEPES, pH 7.5) labeled with the fluorophor bimane (Sandmeier and Christen, 1984) results in a decrease in the relative fluorescence intensity (▲). The maximum decrease at saturating concentrations of aspartate is 32% of the initial value. Excitation was at 380 nm and emission was measured at 470 nm (Sandmeier and Christen, 1984).

inorganic phosphate whereas the binding is only weak ($K_d > 5 \text{ mM}$) in 50 mM sodium phosphate, pH 7.5, as determined by equilibrium dialysis and fluorescence titration (unpublished results).

3.7. Sodium Borohydride and Sodium Cyanoborohydride as Inhibitors of ^3H Release

Addition of borohydride (10 mM) immediately stops the release of ^3H from enzyme-bound [^3H]PMP (Fig. 12). The inhibition is reversible; after ~ 100 min ^3H is released again. At no time during the experiment, transaminase activity as measured after 3000fold dilution of borohydride is found to be affected. For obvious reasons it is not possible to measure transaminase activity in the presence of borohydride. The same inhibition of ^3H release was observed on addition of cyanoborohydride (10 mM). Neither borohydride nor cyanoborohydride impaired the reconstitution of the apoenzyme with PMP (Fig. 12). After reconstitution in the presence of borohydride, pyridoxal (15 mM), dehydroascorbate (20 mM), or NAD^+ (15 mM) were added in order to consume rapidly the borohydride. Immediately, the release of ^3H started with a rate similar to that observed in experiments without borohydride. A control experiment showed that unbound [^3H]PMP did not release measurable quantities of ^3H within 120 min in the presence of pyridoxal, dehydroascorbate, or NAD^+ .

In order to determine the rate of autohydrolysis of borohydride, 10 μl -samples of a 10 mM solution in 50 mM HEPES, pH 7.5, 25°C, were added in timed intervals to a solution of PLP (165 μM in 600 μl 50 mM HEPES, pH 7.5) and the change in absorbance at λ_{max} 388 nm was measured (Peterson and Sober, 1954). Following autohydrolysis over 5 min a half life of ~ 1 min was found which remained unchanged when borohydride was kept in the presence of 0.5 mM aspartate or 39 μM holoenzyme in the PMP form.

In contrast to the short half life of borohydride, the complete inhibition of ^3H release persists for ~ 100 min (see above). The inhibition is not due to longer-lived degradation products of borohydride produced in bulk solution: If sodium borohydride (5 mM in 50 mM HEPES, pH 7.5) is kept at room temperature for

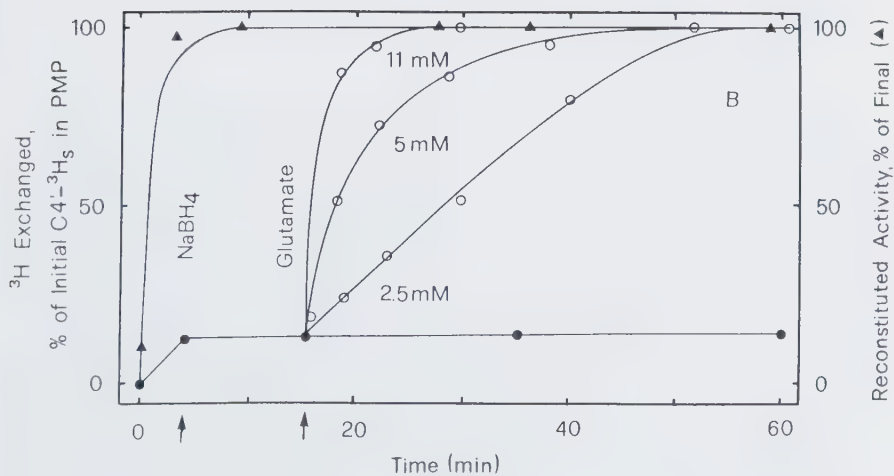
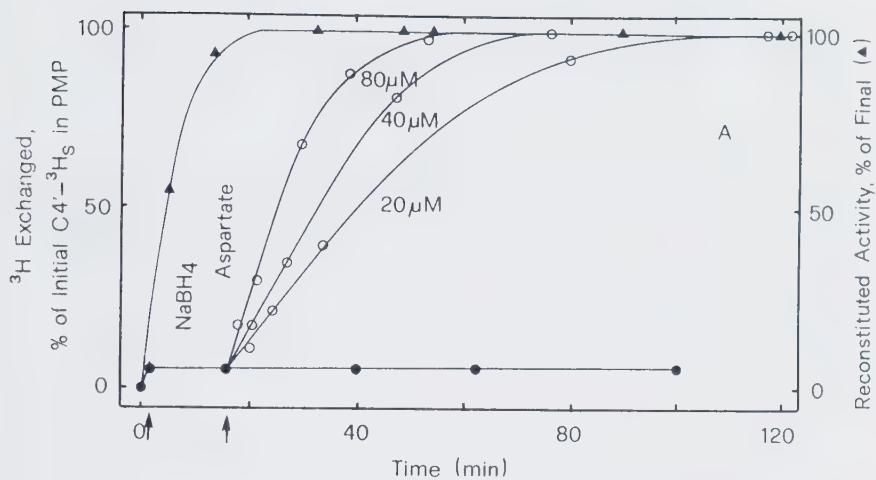


Fig. 12: Inhibitory effect of borohydride ion on the exchange of pro-S ^3H at C4' enzyme-bound PMP and release of this inhibition by aspartate or glutamate. [^3H]PMP (38 μM , total radioactivity 4.6×10^6 cpm μmol^{-1}) was added to apoenzyme in equimolar concentration in 50 mM HEPES, pH 7.5, at room temperature. The binding of coenzyme was followed by removing samples at the indicated times and assaying them for aspartate aminotransferase activity ($\blacktriangle$, 100% corresponded to ~ 230 units mg^{-1}).

(A) Addition of 10 mM sodium borohydride (stock solution in 5 mM NaOH) 1 min after initiation of the reaction (first arrow on time axis) immediately stopped the release of ^3H ($\bullet$) almost completely, while not interfering with reconstitution and enzymic activity (after 3000fold dilution). Addition of aspartate (second arrow on time axis) counteracts the inhibitory effect of sodium borohydride ($\circ$). The final maximum amount (100%) of sublimable radioactivity was 1.9×10^6 cpm μmol^{-1} .

(B) Borohydride was added after completion of the reconstitution (first arrow on time axis) and subsequently glutamate was added (second arrow on time axis).

30 min and then added to [^3H]PMP enzyme (30 μM in 50 mM HEPES, pH 7.5) the rate of ^3H release remains unchanged. The same holds true for 25 mM borate, pH 5.3 - 9.5 (see Fig. 8). Conceivably, borohydride and cyanoborohydride or derivatives thereof form relatively stable complexes with amino or hydroxyl groups, e.g. of PMP, Lys 258 or Tyr 70, thus preventing ^3H release either directly or by interfering with the conformation of the enzyme.

The inhibitory effect of borohydride is partially cancelled by aspartate or glutamate. After stopping ^3H exchange by addition of 10 mM sodium borohydride, both aspartate and glutamate were found to re-initiate ^3H release in a concentration-dependent manner (Fig. 12). ^3H Release in the presence of aspartate in saturating concentration and 10 mM borohydride is, however, still 25 times slower than in the absence of borohydride. The antagonistic effect of aspartate is not due to consumption of borohydride: PMP-holoenzyme (36 μM) was kept for 4 min in the presence of 5 mM aspartate and 10 mM sodium borohydride. The subsequent addition of 15 μM 2-oxoglutarate resulted in an

immediate decrease of the enzymic activity by 38% of its initial value. This loss in activity reflects the reduction of the internal aldimine formed on addition of the oxo acid and indicates the presence of reducing borohydride.

3.8 Release of ^3H and the Conformation of the Enzyme

The observed ^3H release apparently reflects an active site top-chemistry poised to promote deprotonation of the pro-S hydrogen on C4' of PMP which, however, is not yet fully operative in the unliganded enzyme. The system might become activated on formation of the ketimine intermediate or perhaps already on formation of the adsorption complex with the substrate, thus facilitating the formation of the ketimine (see Section 4). The activating effect of the (nonproductive) substrates aspartate and glutamate are consistent with this notion. The following experiments with substrate analogs were performed in order to delineate the relationship between ligand-induced changes in enzyme conformation and ^3H release from [^3H]PMP.

In contrast to the substrates aspartate and glutamate, the substrate analogs 2-methylaspartate and maleate inhibit very strongly the release of ^3H (Table III). The binding of these ligands to the active site results in a shift in the conformational equilibrium of the enzyme. Aspartate aminotransferase is an α_2 dimer consisting of a large coenzyme-binding domain and a small domain. X-Ray crystallographic analysis has defined an open and a closed conformation (Kirsch et al., 1984). In the closed conformation the small domain has moved closer to the active site and thus has narrowed the active site cleft. In mitochondrial aspartate aminotransferase the small domain movement can be monitored by - among other methods - the concomitant changes in reactivity of Cys 166 (Gehring and Christen, 1978). Cys 166 is located 25 Å outside the active site on the

opposite side of the subunit at the tip of the large coenzyme-binding domain close to the boundary to the small domain (Ford et al., 1980).

In the present study, changes in the reactivity of Cys 166 in the PMP form of the enzyme were estimated by following its reaction with 5,5' -dithiobis-(2-nitrobenzoate) spectrophotometrically (Gehring and Christen, 1978). The measurements indicated that the binding of maleate to the PMP form of the enzyme brings about a shift toward the closed conformation whereas aspartate, 2-methylaspartate, and *erythro*-3-hydroxyaspartate have only slight effects (Table III).

The effect of borohydride was assessed by means of the modification with N-ethylmaleimide because photometric measurements are impeded by the gas bubbles generated during autohydrolysis of borohydride. The reaction of Cys 166 with N-ethylmaleimide was followed by the resulting decrease in enzymic activity (maximally 17% of the initial value; Gehring and Christen, 1978). In the presence of sodium borohydride (10 mM initial concentration), N-ethylmaleimide does not inactivate the enzyme. A control experiment with 2-mercaptoethanol (0.1 mM) and N-ethylmaleimide (60 μ M) showed that sodium borohydride (10 mM) does not directly interfere with the modification of sulfhydryl groups (the reaction was followed photometrically, $\epsilon_{300} = 0.62 \text{ mM}^{-1} \text{ cm}^{-1}$; Friedmann, 1952). Apparently, in the presence of borohydride, Cys 166 is not reactive toward N-ethylmaleimide indicating a pronounced shift of the conformational equilibrium toward the open conformation. Modification starts not before ^3H release sets in again, i.e. ~ 100 min after addition of sodium borohydride (see above). ^3H Release and modification with N-ethylmaleimide correspond with each other not only in the temporal course of their spontaneous restoration after borohydride addition but also in the antagonistic effect of aspartate on the inhibition by borohydride (Table III).

TABLE III: Effects of unproductive substrates, substrate analogs, and borohydride on ^3H release from $[^3\text{H}]\text{PMP}$ and the conformation of aspartate aminotransferase. The release of ^3H at the indicated concentrations of ligands was followed as in Fig. 6. The modification of the PMP form of the enzyme ($11\mu\text{M}$) with 0.4 mM 5,5'-dithiobis-(2-nitrobenzoate) in 50 mM HEPES, pH 7.5, was followed photometrically at 412 nm (Gehring and Christen, 1978). The reaction of PMP enzyme ($17\mu\text{M}$) with 0.18 mM N-ethylmaleimide was followed by measuring the ensuing decrease of enzymic activity (maximally 17 % of the initial value, Gehring and Christen (1978)). The latter reagent was chosen for assessing the effect of borohydride because photometric measurements are impeded by gas bubbles generated during its spontaneous hydrolysis.

Ligand	Concentration ^a (mM)	Relative rate ^b of ^3H release	Relative rate ^b of modification of Cys 166	
			Nbs ₂ ^c	NEM ^c
None		1.00	1.00	1.00
Aspartate	6	430	1.1	16
Glutamate	10	400 ^d	1.0	
2-Methylaspartate	35	0.04	1.5	
erythro-3-Hydroxyaspartate	20	0.77	1.35	
Maleate	20	0.033	3.2	4.4
NaBH ₄	10	0 ^e		0 ^e
NaBH ₄ / Aspartate	10/6	4 ^f		2.1

^a 2-Methylaspartate, and erythro-3-Hydroxyaspartate at the concentrations used induce the closed conformation of the PLP form of aspartate aminotransferase in 50 mM sodium phosphate, pH 7.5 (Gehring and Christen, 1978). The concentrations of aspartate, glutamate, and maleate correspond at least to the 10fold of K_m and K_d , respectively.

^b The rates were standardized on the basis of the unliganded PMP form of the enzyme.

^c Nbs₂, 5,5'-dithiobis-(2-nitrobenzoate); NEM, N-ethylmaleimide.

^d Maximum value measured at 10 mM glutamate. Higher concentrations of glutamate inhibit ^3H release.

^e Below detection limit.

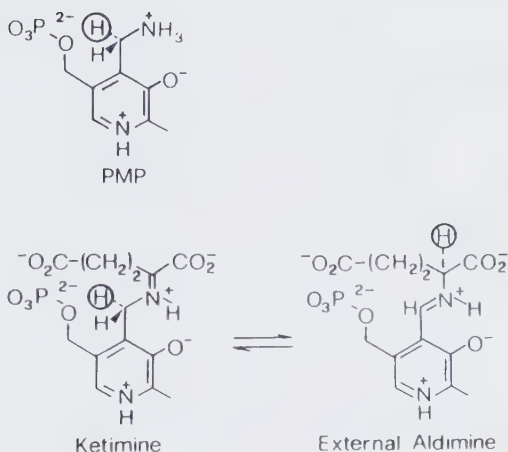
^f Aspartate $80\mu\text{M}$ (see Fig. 12A).

4. Discussion

The present data show that upon binding of PMP to apoaspartate aminotransferase its pro-S hydrogen at C4' exchanges with solvent water. The same hydrogen is involved in the 1,3-prototropic shift from the ketimine to the aldimine intermediate during the enzymic transamination reaction. However, in the present instance the stereospecific labilization occurs independently of the formation of the ketimine intermediate. (The structures of PMP and the tautomerizing intermediates formed with glutamate as substrate are depicted in Scheme II.) The first-order kinetics of the exchange can be clearly recognized because of the fast binding of PMP to phosphate-free apoenzyme.

The occurrence of this exchange may explain the experimental difficulties encountered in detecting proton transfer from C α of the substrate to C4' of the coenzyme during the aldimine-ketimine tautomerization in the aspartate aminotransferase reaction. Such a transfer was first observed by Ayling et al. (1968) with pyridoxamine-pyruvate aminotransferase where pyridoxamine and pyridoxal are themselves substrates and dissociate from the enzyme. Transfer in aspartate aminotransferase (mitochondrial isoenzyme from chicken) where PMP and PLP are two interconvertible forms of the coenzyme which are not released during the reaction, was only recently described (Gehring, 1984). The relatively low percentage of transfer ($\sim 13\%$ of ^3H released from the α -position of glutamate) and the variation of this value in different experiments, was tentatively ascribed at least in part to an exchange of the pro-S hydrogen of enzyme-bound PMP with solvent. In view of the present data, this interpretation seems to be correct and might perhaps also explain the reported failure to detect the transfer in the cytosolic isoenzyme from pig (Dunathan, 1971).

SCHEME II



Several effects may contribute to the labilization of the pro-S hydrogen in enzyme-bound PMP (for the detailed features of the active site and the hypothetical mechanism of action of aspartate aminotransferase, see Kirsch et al., 1984):

(1) The electron withdrawing effect of the protonated N1 of PMP is increased by the formation of a hydrogen bond with Asp 222. (2) An additional contribution might be brought about by the positively charged hydrogen bond system between N4' of PMP and the ϵ -amino group of Lys 258. The fact that no ^3H is released from enzyme-bound [^3H]PMP is consistent with an essential role of the 4' amino group. (3) Other effects must be responsible for the stereospecificity of the deprotonation, the most important one probably being a properly situated proton abstracting group. Very likely, the same group that transfers the proton in the prototropic shift from the

ketimine to the aldimine intermediate (Scheme II) promotes also the deprotonation of PMP. This group has not yet been unequivocally identified. Lys 258 appears to be a good candidate but the participation of Tyr 70 or an intervening water molecule cannot be excluded (for a more detailed discussion, see Kirsch et al., 1984; Gehring, 1984). Enzyme carbamylated at Lys 258 can still perform, although very slowly and irreversibly, a single turnover conversion from its PLP form to the PMP form in the presence of glutamate. If this reaction was carried out in D₂O, PMP was stereospecifically deuterated at the pro-S position of C4' (Martinez-Carrion et al., 1979). The present results suggest that the deuteration might have occurred not only during the tautomerization step but also later in the PMP enzyme. Thus, the results of Martinez-Carrion et al. (1979) provide a serious argument against Lys 258 labilizing the C4'-pro-S hydrogen in the PMP enzyme.

The mechanism of the increase in rate of ³H release on binding of aspartate is open to conjecture. Conceivably, an electrostatic effect of the positive charge of the protonated α -amino group might facilitate the release of the proton. Alternatively, the same effect could be brought about by formation of a salt/hydrogen bond between the α -amino group and the 3' hydroxyl group of the coenzyme. However, the accelerated ³H release might also relate to the change in conformation accompanying the formation of the unproductive complex with aspartate. The labilization of the pro-S hydrogen at C4' of PMP might just reflect an active site configuration poised for the deprotonation of the ketimine intermediate that, however, is not yet fully operative prior to the formation of the Michaelis adsorption complex or the ketimine intermediate itself. On the other hand, it is conceivable that the labilization of the C4' hydrogen reflects an enhanced nucleophilicity of the amino group of enzyme-bound PMP facilitating the formation of the ketimine intermediate (see Kallen et al., 1985).

The most comprehensive explanation for the observed negative and positive modulations of the rate of ^3H release in the presence of nonproductive substrates, substrate analogs, and borohydride is the supposition that they relate to shifts in the open/closed equilibrium of the enzyme. The inhibitory effect of borohydride and cyanoborohydride appears to be accompanied by a shift toward the open conformation, the activating effect of aspartate and glutamate by a shift toward the closed conformation. A causal connection between the rate of ^3H release and the open/closed conformational equilibrium seems at variance with the inhibitory effects of maleate and 2-methylaspartate which have been found to induce the closed conformation. However, maleate with its rigidly fixed carboxylate groups and 2-methylaspartate with its apolar methyl group might be rather special cases of active site ligands perhaps immobilizing the two domains or preventing access of water molecules.

The acceleration of the stereospecific labilization of the pro-S hydrogen at C4' of PMP on formation of a complex with a (unproductive) substrate supports the notion that the observed release of ^3H corresponds to the abstraction of the same proton in the ketimine-aldimine tautomerization (or perhaps the deprotonation of PMP in the productive enzyme substrate complex facilitating ketimine formation). The conclusion that ^3H release is favored by the closed conformation is consistent with previous studies on the enzyme both in solution and in the crystalline state which have shown that the covalency changes of transamination occur in the closed conformation of the enzyme (for a short review, see Chapter I). The present data suggest that the efficiency of at least part of the catalytic apparatus of aspartate aminotransferase is linked to its closed conformation. Measurement of ^3H release from enzyme-

bound [^3H]PMP thus provides an experimental means to probe particular aspects of the catalytic competence of the enzyme independently of substrate turnover.

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CURRICULUM VITAE

Hans Peter Tobler von Zollikon/ZH, geboren am 24. April 1949
in St. Gallen

1967 - 1971	Kantonales Lehrerseminar Rorschach, Lehrerpapent
1971 - 1973	Reallehrer am Schulhaus Feldli in St. Gallen
1973 - 1978	Universität Zürich, Studium der allgemeinen Botanik
1978	Diplom der Philosophischen Fakultät II der Universität Zürich
1978 - 1983	Hauptlehrer für Chemie und Biologie am Institut Dr. Pfister, Oberägeri
1980	Nachdiplomprüfung in Anatomie und Physiologie des Menschen
1981	Diplom für das höhere Lehramt
1983 - 1986	Universität Zürich, Assistent am Biochemischen Institut, Stipendiat der Janggen-Pöhn Stiftung, Ausführung der Dissertation unter der Leitung von Prof. Dr. Ph. Christen
seit 1986	Naturwissenschaftlicher Beamter beim Bundesamt für Umweltschutz

Während meines Studiums besuchte ich bei folgenden Professoren Vorlesungen, Kurse und Praktika:

Anthropologie,	Anatomie und Physiologie des Menschen: J. Biegert, R. Hunsperger, St. Kubik
Biochemie:	H. Bosshard, Ph. Christen, R. Hummel, J. Kägi
Botanik:	R. Bachofen, C.D.K. Cook, P.K. Endress, H.R. Hohl, R. Parish, D. Rast, O. Rohweder, E. Thomas, H. Wanner
Chemie:	C.H. Eugster, H.R. Oswald, H. Fischer, G. Wagnière

Mathematik:	E. Batschelet, P. Hess, G. Karrer, H. Keller, K. Strebel
Molekular- biologie:	M.A. Billeter, M. Birnstiel, C. Weissmann
Pädagogik:	O. Woodtli, K. Widmer
Paläontologie:	E. Kuhn-Schnyder, H. Rieber
Physik:	E. Brun, W. Kündig
Zoologie:	H. Burla, P.S. Chen, H. Hediger, E. Kubli, H. Kummer, R. Nöthiger, P. Tardent, R. Wehner, V. Ziswiler

